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(54) Title: TNF-ALPHA MUTEINS AND A PROCESS FOR PREPARING THEM (57) Abstract The invention relates to a tumor necrosis factor mutein characterized in that the TNF- α amino acid sequence is mutated, or deleted totally or partially, in the region extending from amino acid position at 101 to 116 in such a way that: either the lectin-like activities are modulated with respect to TNF- α , and/or the toxic activity is reduced with respect to TNF- α , and/or the inflammatory cytokines inducing capacities are modulated with respect to TNF- α , and/or the adhesion molecules inducing capacities are modulated with respect to TNF- α , and/or the metastasis promoting activity is reduced with respect to TNF- α , and/or the half life time is increased with respect to TNF- α and with said muteins being characterized in that they have retained the aforementioned activities; or a pharmaceutically acceptable salt thereof.		

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TNF-ALPHA MUTEINS AND A PROCESS FOR PREPARING THEM.

The present invention relates to TNF- α -muteins, a process for preparing them and their use as active substances in pharmaceutical compositions.

Tumor necrosis factor- α (TNF- α), also known as cachectin, is a multifunctional cytokine produced mainly by activated macrophages. In vitro, it has diverse biological effects (reviewed by Manogue et Cerami, 1988) including killing of transformed cells (Carswell et al., 1975), stimulation of granulocytes and fibroblasts (Old, 1985; Vilchek et al., 1986; Beutler and Cerami, 1987), damage to endothelial cells (Sato et al, 1986), and anti-parasitic effects (Taverne et al., 1984). In vivo, it plays a key role as an endogenous mediator of inflammatory, immune and host defence functions and it is involved in a number of pathological conditions in man and mouse such as septic shock, cachexia, capillary leak syndrome, hemorrhagic necrosis of multiple organs, etc. It is capable of acting independently and in conjunction with other factors affecting a whole plethora of different body functions. These effects can either be beneficial or life-threatening to the host. Some of these effects are direct, others may be mediated via the induction of other secreted factors. The biological effects of TNF- α are mediated via binding to specific cell surface receptors.

The primary structure of human and mouse TNF- α (Pennica et al., 1984; Fransen et al., 1985) and of two different TNF-receptors (p55-TNF-R and p75-TNF-R) has been deduced from the nucleotide sequence of the cloned cDNA (Dembic et al., 1990; Loetscher et al., 1990). Both receptors bind not only TNF- α but also TNF- β or lymphotoxin with high affinity (Schoenfeld et al., 1991). TNF- β is a related lymphocyte product (TNF- α and TNF- β share 32% homology on the amino acid sequence level) that exhibits pleiotropic activities very similar to those of TNF- α . X-ray crystallographic analysis revealed that the tertiary

structure of both molecules is virtually identical except that the TNF- β trimer creates a molecule that is less elongated than the TNF- α trimer and the latter has a top-region that flares open (Eck et al., 1992).

Besides the cell receptor interaction, TNF- α has been shown to have a lectin-like property for the oligosaccharide ligands chitobiose and Man(α 1,3)-(Man(α 1,6)-Man (Hession et al., 1987; Sherblom et al., 1988). They further demonstrated that the TNF- α protein has at least two different binding sites, one lectin-like and the other directed at cell surface receptors. Several TNF- α mutants were described for which the binding to the cellular TNF-receptor p55 and/or p75 was hampered. All these mutations are located in the lower half of the pyramidal structure of the biologically active TNF- α trimer (Van Ostade et al., 1991; Van Ostade et al., 1992; EP-A-O 486 908).

The availability of recombinant TNF has enhanced its use in tumor therapy. However, the *in vivo* tumoricidal effects of TNF- α have always been accompanied by toxic side effects. Different approaches were followed to overcome these noxious effects, including the use of monoclonal antibodies as well as fragments thereof, which neutralize the *in vitro* and *in vivo* toxic properties of TNF- α (e.g. EP-A-O 350 690).

In addition to its immunomodulating activity, TNF- α has also been shown to be involved in the control of growth and differentiation of various parasites. Upon infection of the host, parasites are capable of inducing the secretion of different cytokines such as TNF which may affect the course of the disease. For instance, in the case of malaria, TNF- α can be protective in certain circumstances, such as inhibiting parasite survival in rodent malaria (Clark et al., 1987; Taverne et al., 1987). By contrast, its overproduction can be detrimental to the host and can contribute to the pathology of the disease (Clark, 1987; Grau et al., 1989).

In schistosomiasis, the parasite uses the host-derived immunoregulatory protein TNF- α as a signal for replication and transmission (Amiri et al., 1992). Moreover, the authors found that the parasite worms required TNF- α for egg laying and for

excretion of eggs from the host. In cutaneous leishmaniasis, TNF- α plays a protective role (Titus et al., 1989; Liew et al., 1990).

African trypanosome species are the etiological agents for African trypanosomiasis, or sleeping sickness, and are a major cause of both human disease (Trypanosoma brucei rhodesiense and T. brucei gambiense) and cattle disease (T. brucei brucei, T. evansi, T. vivax, T. congolense) in Africa. Injected into their mammalian hosts by tsetse flies, trypanosomes remain extracellular bloodstream trypomastigotes throughout their mammalian phase. In trypanosomiasis, it has been shown that rabbits infected with Trypanosoma brucei brucei developed cachexia and suppression of lipoprotein lipase levels (Rouzer and Cerami, 1980). These parameters are indicators of TNF- α bioactivity (Beutler et al., 1985), thus suggesting that the parasite infection induced TNF *in vivo*. Kongshavn et al. (1988) reported that TNF- α inhibited the growth of *in vitro* cultures of Trypanosoma musculi, but administration of TNF- α *in vivo* during the course of infection led to a net increase in parasite development. The *in vitro* data indicate that a direct interaction between TNF- α and T. musculi can take place. All these experiments point to a direct interaction between host-derived TNF- α and parasites influencing the course and the outcome of parasitemia.

Considerable evidence implicates TNF- α is the principle mediator of endotoxic or septic shock (Cerami and Beutler, 1988). The intravenous injection of LPS (or endotoxin) in animals produces several parameters of the septic shock syndrome, namely, hypotension, decreased systemic vascular resistance, leukopenia, thrombocytopenia, and tissue damage. In experimental animals, TNF- α produces hypotension, leukopenia, and local tissue necrosis (Okusawa et al., 1988). Administration of anti-TNF- α antibodies to baboons (Tracey et al., 1987) or rabbits prevents the shock induced by endotoxin (Mathison et al., 1988).

The term "TNF-induced septic shock" refers to the shock that develops in the presence of severe infection, especially following bacteremia with Gram-negative bacteria and release of

endotoxin. It may also be caused by any class of microorganism, including Gram-positive bacteria, viruses, fungi, protozoa, spirochetes and rickettsiae. The pathophysiology of septic shock is very complex (see Harrison's Principles of Internal Medicine, 12th ed, McGraw-Hill, New York, pp. 502-507). Death is caused by progression to multiple organ failure and circulatory collapse.

Sherblom et al., (1988) have shown that the TNF- α has a lectin-like affinity for the glycoprotein uromodulin. Interestingly, this glycoprotein is able to potently inhibit the TNF-induced shock in vivo, whereas it does not block the tumoricidal activity of TNF- α on L929 cells in vitro. The authors suggested therefore that the lectin-like region of TNF- α is implicated in the toxic effects of TNF- α in septic shock, but not in its tumoricidal activity.

The aim of the present invention is to provide TNF- α muteins which have altered lectin-like properties in comparison with those of TNF- α , and/or TNF- α muteins which have a reduced toxic activity in comparison to TNF- α , and/or TNF- α muteins which have altered inflammatory cytokines inducing capacities with respect to TNF- α , and/or TNF- α muteins with reduced metastasis promoting activities with respect to TNF- α , and/or TNF- α muteins which have an extended half-life time with respect to TNF- α .

The aim of the present invention is to provide TNF- α muteins which have reduced lectin-like properties in comparison with those of TNF- α , and/or result in reduced cachexia or septic shock upon treatment with toxic concentrations of TNF- α in cytokine therapy, without having lost their tumoricidal activity.

Another aim of the present invention is to provide pharmaceutical compositions comprising said TNF- α muteins.

The aim of the present invention is also to provide antisense peptides or antibodies which recognize the TNF- α lectin-like epitope for use in the preparation of a medicament for treating endogenous toxic concentrations of TNF- α due to sepsis, septic shock, Gram negative sepsis, endotoxic shock, toxic shock syndrome, cachexia, microbial infections, rheumatoid arthritis, inflammatory conditions, respiratory distress syndrome, pulmonary fibrosis, infections, graft-versus-host-

disease, reperfusion damage such as myocardial ischaemia, AIDS, cancer, cerebral malaria, immunosuppression, etc.

Another aim of the present invention is to use said antibodies or said antisense peptides, in addition to complete TNF- α , for the preparation of a medicament for treating tumors, with said medicament having reduced toxic side effects and/or altered (i.e. reduced or increased) lectin-like effects and/or altered inflammatory cytokines inducing capacities, and/or altered adhesion molecules inducing capacities, and/or an increased half-life with respect to TNF- α in the absence of said antibodies or antisense peptides.

The present invention relates more particularly to a tumor necrosis factor mutein characterized in that the TNF- α amino acid sequence is mutated, or deleted totally or partially, in the region extending from amino acid position at 101 to 116 in such a way that:

- either the lectin-like activities are modulated with respect to TNF- α ,
 - and/or the toxic activity is reduced with respect to TNF- α ,
 - and/or the inflammatory cytokines inducing capacities are modulated with respect to TNF- α ,
 - and/or the adhesion molecules inducing capacities are modulated with respect to TNF- α ,
 - and/or the metastasis promoting activity is reduced with respect to TNF- α ,
 - and/or the half life time is increased with respect to TNF- α ,
- and providing that these TNF- α muteins have preferentially retained the tumoricidal activity of TNF- α , and providing that said TNF muteins are different from human TNF- α wherein amino acids 1 to 8 are replaced by a sequence within the region spanning amino acids 5 to 30 of laminin, and providing that said TNF muteins are different from human TNF- α wherein amino acid position 101 is Ser, hTNF- α wherein amino acid position 102 is Arg or deleted, hTNF- α wherein amino acid position 103 is Trp, hTNF- α wherein amino acid position 105 is Pro, hTNF- α wherein amino acid position 105 is Ile, hTNF- α wherein amino acid position 105 is Ile and position 44 is Cys, hTNF- α wherein amino

acid position 106 is Ser, hTNF- α wherein amino acid position 106 is Ser and position 131 is Cys, hTNF- α wherein amino acid position 108 is Phe, hTNF- α wherein amino acid position 110 is Lys, hTNF- α wherein amino acid positions 111 to 112 are deleted, hTNF- α wherein amino acid position 112 is deleted or Met, hTNF- α wherein amino acid position 111 is deleted and amino acid positions 109 and 120 are respectively Gln and His, hTNF- α wherein amino acid position 115 is Ile or Cys, hTNF- α wherein amino acid position 116 is Lys, His or Val, hTNF- α wherein amino acid positions 115-116 are Ile-Lys;

and with said TNF muteins possibly containing in their peptidic chain outside the region spanning amino acids 101 to 116 of TNF- α , additional modifications consisting of substitutions and/or deletions and/or additions of one or several amino acid residues, and with said muteins being characterized in that they have retained the aforementioned activities; or a pharmaceutically acceptable salt thereof.

It is to be understood that the TNF- α muteins according to the present invention are characterized in that they have at least one of the following effects:

- lectin-like activities which are modulated (i.e. reduced or increased), preferably reduced, from 5 to 100%, preferably at least 25%, more preferably at least 50%, most preferably at least 75%, as compared with lectin-like activities observed for TNF- α ;
or,

- a toxic effect which is reduced from 5 to 50%, preferably at least 10%, more preferably at least 25%, most preferably at least 35% as compared with the toxic effect of TNF- α ;
or,

- inflammatory cytokines inducing capacities which are modulated (i.e. either reduced or increased), preferably reduced, from 5 to 100%, preferably at least 25%, more preferably at least 50%, most preferably at least 75% with respect to TNF- α ;
or,

- adhesion molecules inducing capacities which are modulated (i.e. either reduced or increased), preferably reduced, from 5 to 100%, preferably at least 25%, more preferably at least 50%,

most preferably at least 75% with respect to TNF- α ;

or,

- a metastasis promoting activity which is reduced from 5 to 100%, preferably at least 25%, more preferably at least 50%; most preferably at least 75% with respect to TNF- α ;

or,

- a half life time that is increased from 5 to 100%, preferably at least 25%, more preferably at least 50%, most preferably at least 75% with respect to TNF- α ;

or,

- the tumoricidal activity of TNF- α being preferentially retained by said muteins.

The tumoricidal activity is considered to be retained within the meaning of the present invention when said TNF- α muteins have a tumoricidal activity of at least 75%, preferably at least 85%, more preferably at least 95% and even more preferably at least 100% of the tumoricidal activity of TNF- α which has not been mutated in the region spanning amino acids 101 to 116. It is, however, also possible that TNF-muteins according to the present invention have largely lost the tumoricidal activities of TNF- α , whilst in addition having at least one, or preferably a combination, of any of the aforementioned activities of the TNF muteins according to the invention which are modulated (i.e. increased or reduced). For instance, TNF-muteins which have increased lectin-like activities and/or a reduced toxicity, and/or a reduced metastasis promoting activity, and/or an extended half life, and which can no longer be classified as having a tumoricidal activity are also contemplated within the present invention.

It is also to be understood that the TNF-muteins according to the present invention will mostly show a combination of at least two of the aforementioned effects. Illustrations of preferred TNF-muteins according to the present invention are given below and in the Examples section.

The term "toxic activity" refers to the in vivo TNF- or LPS-induced septic shock effect of TNF- α as explained above, and may be measured in vivo on the basis of the increased survival time

of C57BL/6 mice, upon injection with fixed dosages of the TNF- α mutein, as compared with that in C57BL/6 mice injected with the same dosages of unmodified TNF- α ; or by any other technique known in the art.

The term "tumoricidal activity" refers to the in vivo tumor cell killing activity of TNF- α , which may be measured by means of any assay comprised in the art. The term "cytotoxic or cytolytical activity" refers to the in vitro tumor cell killing activity of TNF- α . In the Examples section of this application an in vitro assay is reported to measure the cytotoxic activity of TNF- α on L929 TNF-sensitive fibrosarcoma cells. An in vivo assay to evaluate the tumoricidal activity of TNF- α on HT-29 tumors in nude mice, and by means of the B16Bl6 melanoma test system, are also reported in the Examples section.

The term "lectin-like activities" of the present invention refers to the lectin-like properties of TNF- α . The lectin-like properties of TNF- α are for instance those described by Hession et al. (1988) and Sherblom et al. (1988) and include any biological effect mediated by any lectin-carbohydrate interaction located in the region extending from amino acids 101 to 116 of TNF- α . The "lectin-like activities" of the TNF- α muteins of the invention may be measured as the percentage of inhibition of trypanocidal activity of the TNF mutein, upon prior incubation of said TNF mutein with the oligosaccharide sugar N,N'-diacetylchitobiose as described in the Examples section; or by means of any other assay comprised in the art.

The term "trypanocidal activity" refers to the trypanocidal activity of TNF- α located in the region extending from amino acid positions 101 to 116 of TNF- α may be measured by means of an in vitro or an in vivo trypanocidal activity assay as extensively explained in the Examples section.

The term "inflammatory cytokines inducing capacities" of TNF α within the meaning of the present invention refers to the stimulation capacity of TNF- α of cytokines such as IL1 α , IL1 β , IL8, TNF, IL6 and other molecules known to have a role in inflammatory processes by endothelial cells, said capacities

being located in the region extending from amino acids 101 to 116 of TNF- α .

The term "adhesion molecules inducing capacities" of TNF α within the meaning of the present invention refers to the stimulation of adhesion molecules capacity of TNF α , such as the stimulation of ICAM-1, ICAM-2, ECAM-1 (E-selectin), P-selectin, Endoglin, VCAM-1, VLA Integrin (β 1-integrin family), CD36, CD9, CD31, GlyCAM-1, which mediate adhesion events necessary to mount inflammatory reactions, said capacities being located within the region extending from amino acid positions 101 to 116 of TNF α .

The term "metastasis promoting activity" within the meaning of the present invention refers to negative in vivo side effects of TNF- α exerted against tumor cells and resulting in a net increase/growth of the number of metastase such as reviewed by Orosz et al., 1993, located within the region extending from amino acid positions 101 to 116 of TNF α .

The term "half-life" refers to the in vivo measured time during which the TNF- α muteins of the invention remain in circulation and are detectable in body fluids.

The inhibition / stimulation of these TNF- α muteins on the inflammatory cytokines inducing capacities of TNF α may be measured in vivo by any technique known in the art, more particularly any of the techniques disclosed in the examples section of the present invention.

The inhibition / stimulation of these TNF- α muteins on the adhesion molecules inducing capacities of TNF- α may be measured in vivo by any technique known in the art.

The inhibition of metastasis promoting activity by these TNF- α muteins may be measured in vivo by any technique known in the art, or as disclosed in the examples section.

The increase of the half life time by these TNF- α muteins may be measured in vivo by any technique known in the art, or as disclosed in the examples section.

The term "TNF- α " refers to polypeptides of human origin containing 157 amino acid residues, as disclosed by Pennica et al. (1984), namely:

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	5	10	15
1	Val Arg Ser Ser Ser Arg Thr Pro Ser Asp Lys Pro Val Ala His		
16	Val Val Ala Asn Pro Gln Ala Glu Gly Gln Leu Gln Trp Leu Asn		
31	Arg Arg Ala Asn Ala Leu Leu Ala Asn Gly Val Glu Leu Arg Asp		
46	Asn Gln Leu Val Val Pro Ser Glu Gly Leu Tyr Leu Ile Tyr Ser		
61	Gln Val Leu Phe Lys Gly Gln Gly Cys Pro Ser Thr His Val Leu		
76	Leu Thr His Thr Ile Ser Arg Ile Ala Val Ser Tyr Gln Thr Lys		
91	Val Asn Leu Leu Ser Ala Ile Lys Ser Pro <u>Cys Gln Arg Glu Thr</u>		
106	<u>Pro Glu Gly Ala Glu Ala Lys Pro Trp Tyr Glu</u> Pro Ile Tyr Leu		
121	Gly Gly Val Phe Gln Leu Glu Lys Gly Asp Arg Leu Ser Ala Glu		
136	Ile Asn Arg Pro Asp Tyr Leu Asp Phe Ala Glu Ser Gly Gln Val		
151	Tyr Phe Gly Ile Ile Ala Leu		

or as disclosed by Marmenout et al. (1985), or by Wang et al. (1985), or Shirai et al. (1985).

The term "TNF- α " of the present invention also refers to polypeptides of mouse origin containing 156 amino acid residues, as disclosed by Fransen et al. (1985) namely:

	5	10	15
1	Leu Arg Ser Ser Ser Gln Asn Ser Ser Asp Lys Pro Val Ala His		
16	Val Val Ala Asn His Gln Val Glu Glu Gln Leu Glu Trp Leu Ser		
31	Gln Arg Ala Asn Ala Leu Leu Ala Asn Gly Met Asp Leu Lys Asp		
46	Asn Gln Leu Val Val Pro Ala Asp Gly Leu Tyr Leu Val Tyr Ser		
61	Gln Val Leu Phe Lys Gly Gln Gly Cys Pro Asp Tyr - Val Leu		
76	Leu Thr His Thr Val Ser Arg Phe Ala Ile Ser Tyr Gln Glu Lys		
91	Val Asn Leu Leu Ser Ala Val Lys Ser Pro <u>Cys Pro Lys Asp Thr</u>		
106	<u>Pro Glu Gly Ala Glu Leu Lys Pro Trp Tyr Glu</u> Pro Ile Tyr Leu		
121	Gly Gly Val Phe Gln Leu Glu Lys Gly Asp Gln Leu Ser Ala Glu		
136	Val Asn Leu Pro Lys Tyr Leu Asp Phe Ala Glu Ser Gly Gln Val		
151	Tyr Phe Gly Val Ile Ala Leu		

or as disclosed by Pennica et al. (1985), which at least contain the peptide sequence Thr Pro Glu Gly Ala Glu (SEQ ID NO 23) in their peptidic chain.

The expression "TNF- α amino acid sequence which is mutated

in the region extending from amino acid position 101 to 116" refers to either deletions, insertions and/or substitutions in the region of amino acid positions 101 to 116 of TNF- α (including both positions 101 and 116). It is to be understood that the result of all of these deletions is such that at least one of the effects mentioned-above, and preferably combinations of at least two such effects of TNF- α , ascribed to the region covering amino acid positions 101 to 116 of TNF- α , are modulated and by preference are strongly reduced or abolished in the case of life-threatening lectin-like effects (toxic effect, inflammatory cytokines inducing activities, adhesion molecules inducing activities), metastasis promoting effects of TNF- α , or are by preference increased in the case of half-life times and beneficial lectin-like activities (such as induction of cytokines or adhesion molecules which are beneficial to the envisaged treatment).

For TNF- β , the above described septic shock or cachexia effects are not found. The term "TNF- β " refers to tumor necrosis factor β or lymphotoxin (Gray, 1987). TNF- β and TNF- α are members of a duplicated gene family which show 30 to 32% homology at the amino acid level and share many biological activities, including the tumoricidal activity against L929 cells. Despite the fact that both cytokines share the same cell-surface receptor (Aggarwal et al., 1985), important differences exist between TNF- α and TNF- β , both with regard to protein sequence and gene regulation. There are also distinct differences in the mode of secretion in that TNF- α is a membrane protein (Kriegler et al., 1988), whereas TNF- β is a secreted protein.

Table I gives an overview of the amino acid substitutions which could be the basis of some of the additional modifications in the TNF- α muteins in the region outside the region extending from amino acid positions 101 to 116 as defined above.

TABLE I

Amino acids	Synonymous groups
Ser	Ser, Thr, Gly, Asn
Arg	Arg, His, Lys, Glu, Gln
Leu	Leu, Ile, Met, Phe, Val, Tyr
Pro	Pro, Ala, Thr, Gly
Thr	Thr, Pro, Ser, Ala, Gly, His, Gln
Ala	Ala, Pro, Gly, Thr
Val	Val, Met, Ile, Tyr, Phe, Leu, Val
Gly	Gly, Ala, Thr, Pro, Ser
Ile	Ile, Met, Leu, Phe, Val, Ile, Tyr
Phe	Phe, Met, Tyr, Ile, Leu, Trp, Val
Tyr	Tyr, Phe, Trp, Met, Ile, Val, Leu
Cys	Cys, Ser, Thr
His	His, Gln, Arg, Lys, Glu, Thr
Gln	Gln, Glu, His, Lys, Asn, Thr, Arg
Asn	Asn, Asp, Ser, Gln
Lys	Lys, Arg, Glu, Gln, His
Asp	Asp, Asn, Glu
Glu	Glu, Gln, Asp, Lys, Asn, His, Arg
Met	Met, Ile, Leu, Phe, Val

It is reported in the example section of the present invention that TNF- α -derived synthetic peptides located in the region extending from amino acid positions 101 to 116 of TNF- α as indicated in the sequences above, and which will be further referred to as the "TNF- α tip peptides", and which can be represented by the following general formula:

$(Y1-Y2-Y3)_n$ -Thr-X1-Glu-X2-X3-Glu-(Y4-Lys-Pro-Trp-Tyr) $_n$ (SEQ ID NO 1), wherein,

- n represents 0 or 1,
- X1 can represent Pro, Ala, Thr, or Gly;
- X2 can represent Gly, Ala, Thr, Pro, or Ser;
- X3 can represent Ala, Pro, Gly, or Thr;

- Y1 can represent Pro or Gln;
- Y2 can represent Lys or Arg;
- Y3 can represent Asp or Glu;
- and, Y4 can represent Leu or Ala

have a direct trypanocidal activity against the pleomorphic bloodstream forms of the African trypanosomes Trypanosoma brucei brucei and Trypanosoma brucei rhodesiense.

It is to be understood that the TNF- α amino acid position numbering in the present application refers to the numbering as given in the sequences above. In this respect, it is to be noted that the region covering amino acids 101 to 116 of human TNF- α corresponds to amino acids 100 to 115 of mouse TNF- α , since in the sequences given above, a space has been introduced in the mouse TNF- α amino acid sequence position 73 in order to align it with the human TNF- α amino acid sequence.

Moreover, it is shown in the examples section that the trypanocidal activity of the TNF- α "tip-region" can be inhibited by preincubation with N,N'-diacetylchitobiose, for which TNF- α has a lectin-like activity (Hession et al., 1987; Sherblom et al., 1988). Therefore, the tip region of TNF- α is shown to be implicated in the lectin-like activities of the cytokine. Since the glycoprotein uromodulin, which binds TNF- α via its lectin-like domain, can inhibit lipopolysaccharide (LPS)-induced septic shock, caused by TNF- α , but can not inhibit the TNF- α tumoricidal effect on L929 cells, the lectin-like region of TNF- α , and consequently the tip-region of TNF- α , are shown by the present inventors to be implicated in the toxic side effects of the cytokine in vivo.

Consequently, as illustrated in the examples section of the present invention, TNF- α muteins (extending from positions 101 to 116 of TNF- α) were constructed in the tip-region which are characterized in that either the lectin-like activities are reduced with respect to TNF- α , or the toxic activity is reduced with respect to TNF- α ; or the half life is increased with respect to TNF- α ; or the metastasis promoting activities are reduced with

respect to TNF- α , or the inflammatory cytokines inducing capacities are reduced with respect to TNF- α , or a combination of these effects are reduced/increased with respect to TNF- α and with said muteins being further characterized in that they have not lost the tumoricidal activity of TNF- α ; as detailed above.

Consequently the present invention relates more particularly to a TNF mutein as defined above, further characterized in that the lectin-like activities are modulated with respect to TNF- α .

According to a preferred embodiment, the present invention relates to a TNF mutein as defined above, further characterized in that the lectin-like activities are reduced with respect to TNF- α .

It is extensively illustrated in the examples section of the present invention that the region spanning amino acids 101 to 116, and more particularly 105 to 110, is involved not only in the trypanocidal but also in the lectin-like activities of TNF- α . The lectin-like activities were measured indirectly via the inhibition of the trypanocidal activity of TNF- α .

According to a possible embodiment, the present invention also relates to a TNF mutein as defined above, further characterized in that the lectin-like activities are increased with respect to TNF- α . TNF muteins having increased lectin-like activities may be beneficial to certain envisaged treatments.

According to another preferred embodiment, the present invention relates to a TNF mutein as defined above, further characterized in that the toxic activity is reduced with respect to TNF- α . It is illustrated in the examples section of the present invention that mutations to an Ala at positions T(105), E(107) and/or E(110) and a deletion of the region extending from amino acids 105 to 110 of mTNF α leads to mTNF- α muteins with a lower LD50 value as measured in vivo.

According to yet another embodiment, the present invention relates to a TNF mutein as defined above, further characterized in that the inflammatory cytokine inducing capacities are modulated with respect to TNF- α .

According to a preferred embodiment, the present invention

relates to a TNF mutein as defined above, further characterized in that the inflammatory cytokine inducing capacities are increased with respect to TNF- α . As shown in the examples section of the present invention, mTNF- α muteins bearing a deletion of the region spanning amino acids 105-110 of mTNF- α show a higher induction of human interleukin-6 on human microvascular endothelial cells of the brain in vivo than wild-type mTNF α .

According to another possible embodiment, the present invention relates to a TNF mutein as defined above, further characterized in that the inflammatory cytokine inducing capacities are reduced with respect to TNF- α . It is shown in the examples section that T(105)A and P(106)A mTNF- α muteins as well a deletion mutant of mTNF α in the region extending from amino acids 105 to 110 induce consistently less human interleukin 8 (IL8) than wild-type TNF- α on human endothelial cells as measured in vivo.

According to yet another embodiment, the present invention relates to a TNF mutein as defined above, further characterized in that the adhesion molecule inducing capacities are modulated with respect to TNF- α .

According to another preferred embodiment, the present invention relates to a TNF mutein as defined above, further characterized in that the adhesion molecule inducing capacities are reduced with respect to TNF- α .

According to another possible embodiment, the present invention refers to a TNF mutein as defined above, further characterized in that the adhesion molecule inducing capacities are increased with respect to TNF- α .

According to yet another preferred embodiment, the present invention relates to a TNF mutein as defined above, further characterized in that the metastasis promoting activity is reduced with respect to TNF- α . As illustrated in the examples section, a mTNF α mutein carrying a deletion of the region extending from amino acids 105 to 110 shows a considerably lower in vivo metastasis enhancing potential compared to wild-type mTNF α in a murine lung carcinoma cell line model.

According to a preferred embodiment, the present invention relates to a TNF mutein having at least one of the aforementioned effects, further characterized in that the tumoricidal activity is retained with respect to TNF- α . As shown in the examples section, all up till now constructed mTNF α muteins have retained their tumoricidal activity in comparison to wild-type mouse TNF α .

According to yet another possible embodiment of the present invention, any of the TNF muteins having at least one of the effects as defined above are further characterized in that their tumoricidal activity is reduced with respect to TNF- α .

In view of possible treatments with the TNF- α muteins on the basis of their altered lectin-like, or inflammatory cytokines inducing, or adhesion molecules inducing properties, the loss of the tumoricidal effect by certain TNF muteins of the invention may be desirable.

According to yet another preferred embodiment, the present invention relates to a TNF mutein as defined above, further characterized in it shows an increased half life time with respect to TNF- α . As shown in the examples section, the tip deleted (amino acids 105-110) mTNF α muteins shows an increased half life as measured by means of the L929 cytotoxic assay and the indirect ELISA compared to wild-type mTNF α .

According to a preferred embodiment, the present invention relates to any TNF mutein as defined above, characterized in that at least part of the region extending from amino acid positions 101 to 116 of TNF- α , or the complete region corresponding to amino acid positions 101 to 116 of TNF- α has been deleted, and preferably at least the region covering amino acid positions 105 to 110 has been deleted.

According to the latter preferred embodiment said TNF muteins should be different from

- human TNF- α wherein amino acids 1 to 8 are replaced by a sequence within the region spanning amino acids 5 to 30 of laminin, and providing that said TNF muteins are different from human TNF- α wherein amino acid position 105 is Pro, hTNF- α

wherein amino acid position 105 is Ile, hTNF- α wherein amino acid position 105 is Ile and position 44 is Cys, hTNF- α wherein amino acid position 106 is Ser, hTNF- α wherein amino acid position 106 is Ser and position 131 is Cys, hTNF- α wherein amino acid position 108 is Phe, hTNF- α wherein amino acid position 110 is Lys, and hTNF- α wherein amino acid positions 109 and 120 are respectively Gln and His and amino acid 111 is deleted, and may contain substitutions as set out in Table 1, in the region of TNF- α outside the region spanning from amino acid position 105 to amino acid position 110.

According to a preferred embodiment, the present invention relates to a TNF mutein according to as defined above, characterized in that at least one of the amino acids in the region extending from amino acids 101 to 116 of TNF- α has been mutated or deleted more particularly at least one of the amino acids in the region extending from amino acids 105 to 110.

According to a particular embodiment, the present invention relates to TNF muteins wherein the region between amino acid positions 101 and 116 has been mutated in such a way that the amino acids Thr(105), Glu(107) and/or Glu(110) have been mutated to an Alanine residue, as demonstrated in the examples section. Although the examples given relate to mouse TNF- α muteins, also human TNF- α muteins are contemplated within the present invention.

According to yet another embodiment, the present invention relates to a nucleic acid sequence encoding any of the TNF mutein polypeptides as defined above.

According to yet another embodiment, the present invention relates to a process for the preparation of the TNF mutein polypeptides as defined above, comprising the steps of:

- transformation of an appropriate cellular host with a vector, particularly a plasmid, a cosmid, a phage or a virus, in which a nucleic acid sequence as defined above coding for at least one of the TNF mutein polypeptides as defined above has been inserted (insert) under the control of the appropriate regulatory elements, particularly a promoter recognized by the

polymerases of the cellular host and, in the case of a procaryotic host, an appropriate ribosome binding site (RBS), enabling the expression in said cellular host of said nucleic acid sequence,

- culture of said transformed cellular host under conditions enabling the expression of said insert.

The polypeptides according to the present invention can be prepared via recombinant DNA technology. In order to carry out the expression of the polypeptides of the invention in bacteria such as *E. coli* or in an eukaryotic cell such as *Saccharomyces cerevisiae*, or in cultured vertebrate or invertebrate hosts such as insect cells, Chinese Hamster Ovary (CHO), COS1, BHK, and MDCK.

The polypeptides prepared according to this process of the invention will normally contain only naturally occurring L-amino acids. If required, D-amino acids can be incorporated into the polypeptides of the invention according to any technique known in the art, provided their incorporation into said peptides does not interfere with the lectin-like, or toxic, or tumoricidal properties of the TNF muteins according to the invention.

The present invention relates also to TNF muteins characterized in that the TNF- α amino acid sequence is mutated, or deleted totally or partially, in the region extending from amino acid position at 101 to 116 in such a way that:

- either the lectin-like activities are modulated with respect to TNF- α ,
- and/or the toxic activity is reduced with respect to TNF- α ,
- and/or the inflammatory cytokines inducing capacities are modulated with respect to TNF- α ,
- and/or the adhesion molecules inducing capacities are modulated with respect to TNF- α ,
- and/or the metastasis promoting activity is reduced with respect to TNF- α ,
- and/or show an increased half life time with respect to TNF- α , and providing that these TNF- α muteins have preferentially retained the tumoricidal activity of TNF- α ,

and with said TNF muteins possibly containing in their peptidic chain outside amino acid region 101 to 116 of TNF- α , additional modifications consisting of substitutions and/or deletions and/or additions of one or several amino acid residues, and with said muteins being characterized in that they have retained the aforementioned activities; or a pharmaceutically acceptable salt thereof, for treating illnesses and pathological conditions, such as sepsis, septic shock, Gram negative sepsis, endotoxic shock, toxic shock syndrome, cachexia, microbial infections, rheumatoid arthritis, inflammatory conditions, respiratory distress syndrome, pulmonary fibrosis, infections, graft-versus-host-disease, reperfusion damage such as myocardial ischaemia, AIDS, cancer, immunosuppression, cerebral malaria, etc.

It is to be understood that the TNF- α muteins according to the present invention may advantageously be used for the fact that they have a tumoricidal activity, while they lack either the life-threatening toxic, or the lectin-like properties of TNF- α , or the metastasis promoting effects of TNF- α , or the inflammation cytokines inducing capacities of TNF- α or mixture of these effects, and/or because they have an increased half life compared to TNF- α , and/or because they have increased beneficial lectin-like activities and/or inflammatory cytokines inducing activities, and/or adhesion molecules inducing activities compared to TNF- α .

According to an advantageous embodiment, said muteins for treating the above-specified illnesses are preferentially different from human TNF- α wherein amino acids 1 to 8 are replaced by a sequence within the region spanning amino acids 5 to 30 of laminin, and providing that said TNF muteins are preferentially different from human TNF- α wherein:

amino acid position 101 is Ser, hTNF- α wherein amino acid position 102 is Arg or deleted, hTNF- α wherein amino acid position 103 is Trp, hTNF- α wherein amino acid position 105 is Pro, hTNF- α wherein amino acid position 105 is Ile, hTNF- α wherein hTNF- α amino acid position 105 is Ile and position 44 is Cys, hTNF- α wherein amino acid position 106 is Ser, hTNF- α

wherein amino acid position 106 is Ser and position 131 is Cys, hTNF- α wherein amino acid position 108 is Phe, hTNF- α wherein amino acid position 110 is Lys, hTNF- α wherein amino acid positions 111 to 112 are deleted, hTNF- α wherein amino acid position 112 is deleted or Met, hTNF- α wherein amino acid position 111 is deleted and amino acid positions 109 and 120 are respectively Gln and His, hTNF- α wherein amino acid position 115 is Ile or Cys, hTNF- α wherein amino acid position 116 is Lys, His or Val, hTNF- α wherein amino acid positions 115-116 are Ile-Lys.

According to yet another advantageous embodiment of the present invention, the TNF muteins used for treating the above-specified illnesses contain either a mutation of one or more amino acids or a deletion of at least part of the region spanning amino acids 105 to 110 of TNF- α . Particularly preferred examples as well as their biological effects are discussed in the examples section.

According to a preferred embodiment, the present invention relates to a pharmaceutical composition, containing, as active substance, at least anyone of the TNF muteins as defined above, in association with a pharmaceutical acceptable vehicle.

The mode of administration of these pharmaceutical compositions is enteral or preferably parenteral, i.e., intravenous, intraperitoneal, intramuscular, or subcutaneous, with the intravenous route being preferred.

The active substances of these pharmaceutical compositions may be administered alone, without a carrier vehicle; however, they may also be administered with pharmaceutically acceptable non-toxic carriers or diluents, the proportions of which are determined by the suitability and chemical nature of the particular carrier.

According to an advantageous embodiment, the above-mentioned pharmaceutical compositions contain muteins which are preferably different from human TNF- α wherein:

amino acids 1 to 8 are replaced by a sequence within the region spanning amino acids 5 to 30 of laminin, and providing that said TNF muteins are different from human TNF- α wherein amino acid

position 101 is Ser, hTNF- α wherein amino acid position 102 is Arg or deleted, hTNF- α wherein amino acid position 103 is Trp, hTNF- α wherein amino acid position 105 is Pro, hTNF- α wherein amino acid position 105 is Ile, hTNF- α wherein amino acid position 105 is Ile and position 44 is Cys, hTNF- α wherein amino acid position 106 is Ser, hTNF- α wherein amino acid position 106 is Ser and position 131 is Cys, hTNF- α wherein amino acid position 108 is Phe, hTNF- α wherein amino acid position 110 is Lys, hTNF- α wherein amino acid positions 111 to 112 are deleted, hTNF- α wherein amino acid position 112 is deleted or Met, hTNF- α wherein amino acid position 111 is deleted and amino acid positions 109 and 120 are respectively Gln and His, hTNF- α wherein amino acid position 115 is Ile or Cys, hTNF- α wherein amino acid position 116 is Lys, His or Val, hTNF- α wherein amino acid positions 115-116 are Ile-Lys.

According to an advantageous embodiment, the above-mentioned pharmaceutical compositions contain muteins which can be human TNF- α wherein:

amino acids 1 to 8 are replaced by a sequence within the region spanning amino acids 5 to 30 of laminin, and providing that said TNF muteins are different from human TNF- α wherein amino acid position 101 is Ser, hTNF- α wherein amino acid position 102 is Arg or deleted, hTNF- α wherein amino acid position 103 is Trp, hTNF- α wherein amino acid position 105 is Pro, hTNF- α wherein amino acid position 105 is Ile, hTNF- α wherein amino acid position 105 is Ile and position 44 is Cys, hTNF- α wherein amino acid position 106 is Ser, hTNF- α wherein amino acid position 106 is Ser and position 131 is Cys, hTNF- α wherein amino acid position 108 is Phe, hTNF- α wherein amino acid position 110 is Lys, hTNF- α wherein amino acid positions 111 to 112 are deleted, hTNF- α wherein amino acid position 112 is deleted or Met, hTNF- α wherein amino acid position 111 is deleted and amino acid positions 109 and 120 are respectively Gln and His, hTNF- α wherein amino acid position 115 is Ile or Cys, hTNF- α wherein amino acid position 116 is Lys, His or Val, hTNF- α wherein amino acid positions 115-116 are Ile-Lys.

According to yet another advantageous embodiment of the present invention, the TNF muteins used for treating the above-specified illnesses contain either a mutation of one or more amino acids or a deletion of at least part of the region spanning amino acids 105 to 110 of TNF- α . Particularly preferred examples as well as their biological effects are discussed in the examples section.

The present invention relates also to the use of a TNF mutein characterized in that the TNF- α amino acid sequence is mutated, or deleted totally or partially, in the region extending from amino acid position at 101 to 116, more particularly in the region extending from amino acid positions 105 to 110, in such a way that:

- either the lectin-like activities are modulated with respect to TNF- α ,
 - and/or the toxic activity is reduced with respect to TNF- α ,
 - and/or the inflammatory cytokines inducing capacities are modulated with respect to TNF- α ,
 - and/or the adhesion molecules inducing capacities are modulated with respect to TNF- α ,
 - and/or the metastasis promoting activity is reduced with respect to TNF- α ,
 - and/or show an increased half life time with respect to TNF- α ,
- and providing that these TNF- α muteins have preferentially retained the tumoricidal activity of TNF- α , and with said TNF muteins possibly containing in their peptidic chain outside amino acid region 101 to 116 (or respectively 105 to 110) of TNF- α , additional modifications consisting of substitutions and/or deletions and/or additions of one or several amino acid residues, and with said muteins being characterized in that they have retained the aforementioned activities; or a pharmaceutically acceptable salt thereof, for the preparation of a medicament for treating illnesses and pathological conditions, such as, sepsis, septic shock, Gram negative sepsis, endotoxic shock, toxic shock syndrome, cachexia, microbial infections, rheumatoid arthritis, inflammatory conditions, respiratory

distress syndrome, pulmonary fibrosis, infections, graft-versus-host-disease, reperfusion damage such as myocardial ischaemia, AIDS, cancer, immunosuppression, cerebral malaria, etc.

It is to be understood that the TNF- α muteins according to the present invention may especially be used for the fact that they may have a tumoricidal activity, while they lack either the toxic, and/or the lectin-like properties of TNF- α , and/or the metastasis promoting effects of TNF- α , and/or the inflammatory cytokines inducing capacities of TNF α , and/or the adhesion molecules inducing capacities of TNF α , or mixture of these effects, and/or because they have an increased half life compared to TNF- α .

According to another embodiment, the present invention relates to the use of an antibody specifically detecting an epitope residing in the region encompassing amino acids 101 to 116 of TNF- α , preferably in the region encompassing amino acids 105 to 110 of TNF α more particularly a monoclonal antibody, characterized in that it:

- either modulates the lectin-like activities of TNF- α ,
 - and/or inhibits the toxic activity of TNF- α ,
 - and/or modulates the inflammatory cytokines inducing capacities of TNF- α ,
 - and/or modulates the adhesion molecules inducing capacities of TNF- α ,
 - and/or inhibits the metastasis promoting activity of TNF- α ,
- for the preparation of a medicament used for treating TNF- α -induced septic shock, or for treating any pathological condition associated with the in vivo lectin-like activities of TNF- α , such as for treating sepsis, septic shock, Gram negative sepsis, endotoxic shock, toxic shock syndrome, cachexia, microbial infections, rheumatoid arthritis, inflammatory conditions, respiratory distress syndrome, pulmonary fibrosis, infections, graft-versus-host-disease, reperfusion damage such as myocardial ischaemia, AIDS, cancer, immunosuppression, cerebral malaria, etc.

It is to be understood that the antibodies according to the

present invention are characterized in that they have at least one of the following effects:

- they modulate (i.e. inhibit or stimulate), preferably inhibit, the lectin-like effects of TNF- α preferentially from 5 to 100%, preferably at least 25%, more preferably at least with 50%, most preferably with at least 75%;

or,

- they inhibit the toxic effect of TNF- α with from 5 to 50%, preferably at least 10%, more preferably with at least 25%, most preferably with at least 35%;

or,

- they modulate (i.e. inhibit or stimulate), preferably inhibit, the inflammatory cytokines inducing capacities of TNF- α from 5 to 100%, preferably with at least 25%, more preferably with at least 50%, most preferably with at least 75%;

or,

- they modulate (i.e. inhibit or stimulate), preferably inhibit, the adhesion molecules inducing capacities of TNF- α from 5 to 100%, preferably with at least 25%, more preferably with at least 50%, most preferably with at least 75%;

or,

- they inhibit the metastasis promoting activity of TNF- α from 5 to 100%, preferably with at least 25%, more preferably with at least 50%, most preferably with at least 75% with respect to TNF- α .

The inhibition / stimulation of the lectin-like activities of TNF- α by the antibodies of the invention is measured as the percentage of inhibition of trypanocidal activity upon prior incubation of TNF- α with said antibody of the invention in the presence and/or absence of N,N'-diacetylchitobiose; or by any other method known in the art.

The inhibition of the toxic activity of TNF- α by the antibodies of the invention may be measured by the increase in survival time of Balb/C mice, upon treatment with LPS in the presence of different concentrations of the antibody of the invention, as illustrated in the Examples section.

The inhibition of the tumoricidal activity may be measured by means of any assay comprised in the art which measures this activity in vivo on TNF-sensitive tumor cells. In the example section of this application, an in vitro assay to measure the cytotoxic (cytolytic) activity of TNF- α on L929 TNF-sensitive fibrosarcoma cells is reported. An in vivo assay to evaluate the tumoricidal activity of TNF- α on HT-29 tumors in nude mice, and by means of the B16Bl6 melanoma test system, are also reported in the examples section.

The inhibition / stimulation of these antibodies on the inflammatory cytokines inducing capacities of TNF α may be measured in vivo by any technique known in the art, more particularly any of the techniques disclosed in the examples section of the present invention.

The inhibition / stimulation of these antibodies on the adhesion molecules inducing capacities of TNF- α may be measured in vivo by any technique known in the art.

The inhibition of metastasis promoting activity by these antibodies of TNF- α may be measured in vivo by any technique known in the art, or as disclosed in the examples section.

In order to assume inhibition of one or combinations of any of the mentioned effects in vivo, the monoclonal antibody of the invention should form an immunological complex with the subregion of the TNF- α "tip-region" responsible for either of the effects of TNF- α .

According to a preferred embodiment, the present invention relates to the use of an immunological complex, comprising a monoclonal antibody as defined above, and TNF- α , for the preparation of a medicament.

According to this embodiment of the invention, a pharmaceutical composition, containing, as active substance, an immunological complex comprising a monoclonal antibody as defined above, and complete TNF- α , in association with a pharmaceutical acceptable vehicle, is provided.

Instead of, or in addition to TNF- α , the above mentioned compositions could also contain IL-1 α , IL-1 β , IL-6, IFN-gamma,

IFN α/β , IL-8, or any other immunoreactive agent.

The above-mentioned composition can be used for treating illnesses wherein a treatment with $\text{TNF-}\alpha$ is beneficial such as tumors.

Moreover, the above-mentioned composition may especially be used for the fact that it exerts a tumoricidal activity, while lacking either the toxic, and/or the lectin-like properties of $\text{TNF-}\alpha$, and/or the metastasis promoting effects of $\text{TNF-}\alpha$, and/or inflammatory cytokines inducing capacities, and/or adhesion molecules inducing capacities, or mixture of these effects, and/or because they have an increased half life compared to $\text{TNF-}\alpha$. Therefore, they are ideally suited for treating tumors or cancers.

The antibodies according to this embodiment of the invention may include polyclonal or monoclonal antibodies.

The monoclonal antibodies of the invention can be produced by any hybridoma liable to be formed according to classical methods from splenic cells of an animal, particularly of a mouse or rat, immunized against the polypeptides according to the invention, or muteins thereof, or fragments thereof defined above on the one hand, and of cells of a myeloma cell line on the other hand, and to be selected by the ability of the hybridoma to produce the monoclonal antibodies recognizing the polypeptides which has been initially used for the immunization of the animals.

The antibodies involved in the invention can be labelled by an appropriate label of the enzymatic, fluorescent, or radioactive type.

In the case of treatment of human illnesses, the monoclonal antibodies according to this preferred embodiment of the invention may be humanized versions of mouse monoclonal antibodies made by means of recombinant DNA technology, departing from parts of mouse and/or human genomic DNA sequences coding for H and L chains or from cDNA clones coding for H and L chains.

Alternatively the monoclonal antibodies according to this preferred embodiment of the invention may be human monoclonal

antibodies. Such human monoclonal antibodies are prepared by EBV immortalization or, for instance, by means of human peripheral blood lymphocyte (PBL) repopulation of severe combined immune deficiency (SCID) mice (for recent review see Duchosal et al., 1992).

The invention also relates to the use of the peptides or polypeptides of the invention for the selection of recombinant antibodies by the process of repertoire cloning (Perrson et al., 1991).

Monoclonal antibodies can be characterized according to their subclass by known methods such as Ouchterlony immunodiffusion.

It is furthermore known in the art that monoclonal antibodies, or fragments thereof, can be modified for various uses, and fragments can be generated which retain the antigen binding properties. Such fragments are commonly generated by, for instance enzymatic digestion of the antibodies with papain, pepsin, or other proteases.

The peptides according to this embodiment of the invention may be circularized before immunization. The expression "circularized" refers to a joining of the N- and C- terminal cysteine residues by means of oxidation at low molar concentration to promote intramolecular disulfide bridges or by any other technique comprised in the art.

If desired, all above-mentioned peptides or polypeptides can be provided for with an extra N- and C-terminal cysteine residue, in order to circularize the peptide.

The peptides used for immunization are preferably in the form in which they are joined to a biotin molecule, or any other carrier molecule in order to achieve a good immunogenic response. As demonstrated in the Examples section, the biotin molecule may be coupled to the peptide of choice via a linker, such as Gly-Gly, or any other linker comprised in the art.

Furthermore, the peptides used for immunization preferentially contain only naturally occurring L-amino acids. If required, D-amino acids may be incorporated as long as the

immunogenic, lectin-like, toxic and/or trypanocidal properties of the peptides have been retained.

The TNF-induced septic shock effect may be assayed by means of the increase of the survival time after injections of the antibody to LPS-treated Balb/C mice as illustrated in the Examples section.

As mentioned above, the tip region of TNF- α may be implicated in the toxic side effects of the cytokine on cells, thereby limiting the wider therapeutic use of TNF- α on normal cells (reviewed by Taniguchi and Sohmura, 1991). Therefore, antibodies recognizing the tip region of TNF- α can be of particular interest to discriminate between the tumoricidal effect of TNF- α on the one hand, and the trypanolytic and toxic effect of TNF- α on the other. Antibodies recognizing the above-mentioned TNF- α tip region polypeptides of the invention, will be further referred to as anti-tip antibodies.

The present invention relates also to the use of an antisense peptide of a peptide comprising at least part of the region extending from amino acids 101 to 116 of TNF- α , preferably comprising at least part of the region extending from amino acids 105 to 110 characterized in that it:

- either modulates the lectin-like activities of TNF- α ,
- and/or inhibits the toxic activity of TNF- α ,
- and/or modulates the inflammatory cytokines inducing capacities of TNF- α ,
- and/or modulates the adhesion molecules inducing capacities of TNF- α ,

- and/or inhibits the metastasis promoting activity of TNF- α ,
for the preparation of a medicament for treating either the TNF-induced septic shock effect of TNF- α , or for treating pathological conditions associated with the in vivo lectin-like effect of TNF- α , such as for treating sepsis, septic shock, Gram negative sepsis, endotoxic shock, toxic shock syndrome, cachexia, microbial infections, rheumatoid arthritis, inflammatory conditions, respiratory distress syndrome, pulmonary fibrosis, infections, graft-versus-host-disease, reperfusion damage such

as myocardial ischaemia, AIDS, cancer, immunosuppression, cerebral malaria, etc.

It is to be understood that the antisense peptides according to the present invention are characterized in that they have at least one of the following effects:

- they modulate (ie inhibit or stimulate), preferably inhibit, the lectin-like effect of TNF- α preferentially from 5 to 100%, preferably at least 25%, more preferably at least with 50%, most preferably with at least 75%;

or,

- they inhibit the toxic effect of TNF- α with from 5 to 50%, preferably at least 10%, more preferably with at least 25%, most preferably with at least 35%;

or,

- they modulate (i.e. inhibit or stimulate), preferably inhibit the inflammatory cytokines inducing capacities of TNF- α from 5 to 100%, preferably with at least 25%, more preferably with at least 50%, most preferably with at least 75%;

or,

- they modulate (i.e. inhibit or stimulate), preferably inhibit the adhesion molecules inducing capacities of TNF- α from 5 to 100%, preferably with at least 25%, more preferably with at least 50%, most preferably with at least 75%;

or,

- they inhibit the metastasis promoting activity of TNF- α from 5 to 100%, preferably with at least 25%, more preferably with at least 50%, most preferably with at least 75%

All these effects may be measured as mentioned for the antibodies.

More particularly, the term "antisense peptide" is reviewed by Blalock (1990) and by Roubos (1990). In this respect, the molecular recognition theory (Blalock, 1990) states that not only the complementary nucleic acid sequences interact but that, in addition, interacting sites in proteins are composed of complementary amino acid sequences (sense ligand with receptor or sense ligand with antisense peptides).

Thus, two peptides derived from complementary nucleic acid sequences in the same reading frame will show a total interchange of their hydrophobic and hydrophilic amino acids when the amino terminus of one is aligned with the carboxy terminus of the other. This inverted hydropathic pattern might allow two such peptides to assume complementary conformations responsible for specific interaction.

The antisense peptides can be prepared as described in Ghiso et al. (1990). By means of this technology it is possible to logically construct a peptide having a physiologically relevant interaction with a known peptide by simple nucleotide sequence analysis for complementarity, and synthesis of the peptide complementary to the binding site. The antisense peptides according of this preferred embodiment of the invention can be prepared by classical chemical synthesis.

The synthesis can be carried out in homogeneous solution or in solid phase.

For instance, the synthesis technique in homogeneous solution which can be used is the one described by Houbenweyl in the book entitled "Methode der organischen chemie" (Method of organic chemistry) edited by E. Wunsh, vol. 15-I et II. THIEME, Stuttgart, 1974.

The antisense peptides of the invention can also be prepared in solid phase according to the methods described by Atherton and Shepard in their book entitled "Solid phase peptide synthesis" (IRL Press, Oxford, 1989).

In order to assume the inhibition of at least one, or preferably a combination of at least two of the afore mentioned effects in vivo, the antisense peptide should form a complex with the subregion of the TNF- α tip region responsible for either of these effects of TNF- α .

The present invention relates also to the use of a complex consisting of an antisense peptide as defined above, and TNF- α , for the preparation of a medicament.

According to this embodiment of the invention, a pharmaceutical composition, containing, as active substance, a

complex comprising an antisense peptide as defined above, and complete TNF- α , in association with a pharmaceutical acceptable vehicle, is provided.

Instead of, or in addition to TNF- α , the above mentioned compositions could also contain IL-1 α , IL-1 β , IL-6, IFN-gamma, IFN α/β , IL-8, or any other immunoreactive agent.

The above-mentioned composition can be used for treating illnesses wherein a treatment with TNF- α is beneficial such as tumors.

According to a preferred embodiment, the present invention relates to the use of an antibody or an antisense peptide as defined above, characterized in that it modulates the lectin-like activities of TNF- α .

According to an even more preferred embodiment, the present invention relates to the use of an antibody or an antisense peptide as defined above, characterized in that it inhibits the lectin-like activities of TNF- α . Illustrations of said embodiment containing an antibody are given in the examples section of the invention.

According to a possible embodiment, the present invention relates to the use of an antibody or an antisense peptide as defined above, characterized in that it stimulates the lectin-like activities of TNF- α .

According to yet another preferred embodiment, the present invention relates to the use of an antibody or an antisense peptide as defined above, characterized in that it inhibits the toxic activity of TNF- α .

According to yet another embodiment, the present invention relates to the use of an antibody or an antisense peptide as defined above, characterized in that it modulates the inflammatory cytokines inducing capacities of TNF- α .

According to yet another preferred embodiment, the present invention relates to the use of an antibody or an antisense peptide as defined above, characterized in that it inhibits the inflammatory cytokines inducing capacities of TNF- α .

According to yet another embodiment, the present invention

relates to the use of an antibody or an antisense peptide as defined above, characterized in that it modulates the adhesion molecules inducing capacities of TNF- α .

According to yet another preferred embodiment, the present invention relates to the use of an antibody or an antisense peptide as defined above, characterized in that it inhibits the adhesion molecules inducing capacities of TNF- α .

According to yet another possible embodiment, the present invention relates to the use of an antibody or an antisense peptide as defined above, characterized in that it stimulates the adhesion molecules inducing capacities of TNF- α .

According to yet another preferred embodiment, the present invention relate to the use of an antibody or an antisense peptide as defined above, characterized in that it inhibits the metastasis promoting activity of TNF- α ,

According to yet another preferred embodiment, the present invention relates to the use of any of the above-defined antibodies or antisense peptides, characterized in that they increase the half life time of TNF- α . Antibodies or antisense peptides according to this latter embodiment are preferably used in combination with TNF- α .

It is to be understood that the TNF muteins according to the present invention can be administered either directly in the form of mutein protein in a pharmaceutical composition as described above, or in the form of cells transfected with the nucleic acid coding for such muteins, said nucleic acid being inserted into any suitable vector, as known to those skilled in the art.

Thus, the present invention relates also to a method for treating patients suffering from any of the above mentioned illnesses consisting of administering cells transfected with the cDNA coding for the TNF muteins as defined above, said nucleic acid being inserted into any suitable vector, as known to those skilled in the art.

The cells transfected by such a vector-nucleic acid combination are preferably autologous cells derived from the patient (e.g. a cancer patient) to be treated with such

compositions. Said vector-nucleic acid combination has to be constructed in such a way as to allow continuous expression of the mutein protein at either a constant level or at a level which can be modified, depending on the exact nature of the vector used to make the vector-nucleic acid combination. In this format, the present invention may be used to treat cancer patients.

ABBREVIATIONS

TNF- α : tumor necrosis factor-alpha

mTNF- α : mouse tumor necrosis factor-alpha

hTNF- α : human tumor necrosis factor-alpha

Bio: Biotin group

r : recombinant

wt : wild-type

BRIEF DESCRIPTION OF THE FIGURES

Figures 1A and 1B: Restriction map of the plasmid pIG2 used to express the wild type and tip-mutated or tip-deleted mTNF- α .

Figure 2: pIG2 nucleic acid sequence (SEQ ID NO 2). The origin of nucleotide stretches used to construct plasmid pIG2 is specified hereafter.

Position

- | | | |
|----------|---|---|
| 3300-206 | : | lambda PL containing EcoRI-MboII blunt fragment of pBL(λ) (Pharmacia) |
| 207-266 | : | synthetic sequence containing multiple cloning sites and the ribosome binding site of which the ATG initiation codon is located at position 232-234 |
| 267-772 | : | rrnBT ₁ T ₂ containing HindIII-SspI fragment from pKK223 (Pharmacia) |
| 773-3300 | : | tetracycline resistance gene and origin of replication containing the EcoRI-DraI fragment of pAT 153 (Bioexcellence) |

Figures 3A and 3B: In vitro trypanocidal activity of recombinant mouse (m) or human (h) TNF- α on T. brucei brucei (A) and T. rhodesiense (B). Thereto, 2×10^6 /ml purified bloodstream trypanosomes, isolated 1 day before the first in vivo maximum peak, are incubated for 5 hours in PBS, 1% glucose, 1% normal mouse serum at 37 °C with various concentrations of recombinant TNF- α . After 5 hours, the number of living parasites is assessed and is compared with the control wells in which only incubation medium was added. The spontaneous mortality in the control wells was always lower than 10%. The values represent the means of quadruplicates and the standard deviation.

Figure 4: LiCl potentiates the mouse TNF- α mediated trypanolysis in vitro. Trypanosomes were incubated for 5 hours with serial dilution of mTNF- α alone or enriched with 1 μ g/ml of LiCl either in the presence or absence of anti-mTNF- α 1F3F3D4 monoclonal antibodies, which are capable of neutralizing the cytotoxic effect of TNF- α . The values represent the means of quadruplicates and the standard deviation. Especially at low TNF- α concentrations (50 and 500 pg/ml), the values in the LiCl group differed significantly (<0.0008 and 0.009) from the control group indicating that LiCl synergistically potentiates the trypanocidal effect of mTNF- α . LiCl by itself had no effect on the survival of the trypanosomes as no difference with control values were seen (data not shown).

Figures 5A and 5B: T. brucei brucei lysate inhibits the in vitro trypanocidal but not the cytolytic (cytotoxic) capacity of mTNF- α .

Preincubation of mTNF- α for 1 h with 10 μ g/ml of trypanosome lysate results in a potent inhibition of the TNF- α -specific trypanocidal effect (A), whereas it has no effect on the cytolytic or tumoricidal capacity of TNF- α (B). The neutralizing anti-mTNF- α 1F3F3D4 monoclonal antibody (anti-mTNF; Lucas et al., 1990) was used to demonstrate that cytolysis was specifically

indicated by $\text{TNF-}\alpha$. The values represent the means of quadruplicates and the standard deviation.

Figures 6A and 6B: Preincubation of $\text{mTNF-}\alpha$ with N,N' -diacetylchitobiose (indicated as chitobiose) potentially inhibits the trypanocidal activity (A), but not the cytolytic activity (B) of mouse $\text{TNF-}\alpha$. $\text{mTNF-}\alpha$ was preincubated for 2 h with 1 $\mu\text{g/ml}$ of N,N' -diacetylchitobiose before adding it in a serial dilution to bloodstream forms of *T. brucei brucei* (A) or to L929 cells (B) to be tested on its trypanocidal or cytolytic activity (as described in the examples), respectively.

Figure 7: Trypanocidal activity of the circularized, biotinylated mouse $\text{TNF-}\alpha$ tip peptide (Bio- GG-CGPKDTPEGAE LKPWYC) (mtip) and the biotinylated human $\text{TNF-}\alpha$ tip peptide (Bio-GG-CGQRETPEGAEAKPWYC) (htip), and the biotinylated linear subpep1 (Bio-GG-CTPGAEC) (subtip) on the bloodstream forms of *T. brucei brucei* and inhibition of the trypanocidal activity by pre-incubation with N,N' -diacetylchitobiose (Chitobiose). Serial dilutions of these circularized mouse or human $\text{TNF-}\alpha$ tip peptides were added either directly to $2 \times 10^6/\text{ml}$ purified bloodstream trypanosomes or after pre-incubation for 2 h with 1 $\mu\text{g/ml}$ of N,N' -diacetylchitobiose. After 5 hours, the number of living parasites is assessed and is compared with the control wells in which only incubation medium was added.

Figure 8: Trypanocidal activity of different synthetic tip peptides. Serial dilutions of $\text{mTNF-}\alpha$ tip (Bio-GG-CGPKDTPEGAE LKPWYC), $\text{hTNF-}\alpha$ tip (Bio-GG-CGQRETPEGAEAKPWYC), subpep 2 (Bio-GG-EGAE LKPWY), subpep 1 (Bio-GG-C-TPEGAE-C) and the D-amino acid analogue of subpep 1 (=subpep 4) were added to $2 \times 10^6/\text{ml}$ purified bloodstream forms of *T. brucei brucei*.

Figure 9: Trypanocidal activity of subpeptide 1 and muteins thereof. Serial dilutions of subpep 1 (Bio-GG-C-TPEGAE-C), subpep 3 (Bio-GG-TPE), mutpep 1 (Bio-GG-C-APEGAE-C), mutpep 2 (Bio-GG-C-

TAEGAE-C), mutpep 3 (Bio-GG-C-TPAGAE-C), mutpep 4 (Bio-GG-C-TPEAAE-C) and mutpep 5 (Bio-GG-C-TPEGAA-C) made in 100 μ l were added to 2×10^5 /ml purified bloodstream forms of T. brucei brucei (final concentration 10^6 parasites/ml). As in Figure 8, after 5 hours, the number of live parasites is assessed and compared with the control wells in which only incubation medium is added.

Figure 10: The anti-mTNF- α tip polyclonal antibody (anti-tip) potently inhibits the trypanocidal effect of mTNF- α , but it only weakly inhibits the cytolytical activity against L929 cells. mTNF- α was preincubated for 1 h with 10 μ g/ml of protein G purified anti-tip polyclonal IgG before adding it to 2×10^6 bloodstream purified T. brucei brucei/ml. After 5 hours the trypanocidal activity of mTNF- α alone or pretreated with anti-tip polyclonal IgG was evaluated by counting the number of live parasites. The cytolytical activity of TNF- α alone or pretreated with anti-tip polyclonal IgG was evaluated on cell survival of L929 cells after 48 hours incubation in the absence of actinomycin D.

Figure 11: In vitro cytolytic (cytotoxic) activity of wild type mTNF- α and mTNFT105A on L929 cells as tested in the 48 hours assay in the absence of Actinomycin D. The assay was performed as explained in the Materials and Methods section of the Examples.

Figure 12: In vitro T. brucei brucei trypanocidal of wild type mTNF- α and T105A mTNF- α . The trypanocidal activity of wild type and T105A mutated mTNF- α was tested as explained in the legend of figure 3.

Figure 13: Effect of the intravenous (i.v.) injection of different concentrations of wild type and T105A mutated mTNF- α on the survival time of C57BL/6 mice. Per group of 6 mice, 10 μ g (top left), 5 μ g (top right), 2.5 μ g (bottom left) or 1 μ g (bottom

right) per 200 μ l of PBS of either wild type mTNF- α or T105A tip mutated mTNF- α was i.v. injected and the survival time was assessed until 72 hours after injection.

EXAMPLES

Materials and methods

1. Animals

Female 10-week old Balb/C mice (SCK, Mol, Belgium) were used in all *in vivo* LPS-induced septic shock experiments.

Female 10-weeks old C57BL/6 mice (Iffa Credo, France) were used in all *in vivo* TNF-induced lethal shock experiments.

2. Cell lines

L929 fibrosarcoma cells were obtained from the Rega Institute, Leuven, Belgium.

3. Trypanosomes

The *Trypanosoma brucei brucei* AnTat 1.1 (EATRO 1125) pleomorphic bloodstream form was kindly provided to us by Dr. N. Van Meirvenne of the Institute for Tropical Medecine, Antwerp, Belgium, and the *Trypanosoma brucei rhodesiense* Trp 11 pleomorphic bloodstream form was kindly provided to us by Dr. E. Bajyana Songa (Dept. Molecular Biology, Free University of Brussels, Belgium)

A *T. brucei* lysate was prepared by a triple freeze-thaw cycle of purified trypanosomes.

4. Cytokines, antibodies and biochemicals

Purified recombinant mTNF- α and recombinant hTNF- α , produced in *E. coli*, with a specific activity of 10^8 units/mg and 2×10^7 units/mg respectively, were produced by Innogenetics, Gent, Belgium. Human recombinant TNF- β (lymphotoxin) is commercially available from the Genzyme Corporation (Cambridge, MA, USA).

The rat-anti-recombinant mTNF- α 1F3F3D4 (Lucas et al., 1990) and the mouse-anti-recombinant hTNF- α monoclonal antibodies are

both commercially available from Innogenetics (Ghent, Belgium).

The soluble p55 hTNF- α receptor protein was obtained from Dr. D. Wallach (Dept. of Membrane Research & Biophysics, Weizmann Institute, Rehovot, Israel).

Lipopolysaccharide W E. coli 055:B5 (LPS) was purchased from Difco Laboratories, Detroit, MI, USA.

5. Oligosaccharides/lectins

N,N'-diacetylchitobiose was purchased from Sigma (St. Louis, MO, USA). In the in vitro TNF- α -trypanocidal assay, the TNF- α -derived tip peptides are preincubated for 1 h with 1 μ g/ml oligosaccharide.

The lectins Griffonia (Bandeiraea) simplicifolia A4 (GSI-A4) and Triticum vulgaris agglutinin (WGA) were purchased from Sigma (St. Louis, MO, USA).

Galanthus nivalis agglutinin (GNA) and Urtica dioica agglutinin (UDA) were purified and kindly provided by Dr. L. Wijns (Laboratory of Crystallography, Free University of Brussels, Belgium).

6. In vitro biological assay to measure the cytotoxic and trypanocidal activity of recombinant hTNF- α or mTNF- α

6.1. L929 cell killing assay

TNF-sensitive mouse fibrocarcinoma L929 cells in complete RPMI 1640 medium (Gibco BRL, Paisley, Scotland) were treated with actinomycin D (1 μ g/ml) at a cell concentration of 3×10^5 cells/ml. Thus, 100 μ l of the suspension in 96-well flat bottomed culture plates was mixed with 100 μ l, containing either medium (negative control), 100 μ l h/mTNF- α (serially diluted in medium; positive control), or 100 μ l h/mTNF- α + 1 μ g/ml of oligosaccharide, or 100 μ l of the TNF- α derived tip peptides (serially diluted in medium). After 18 h of culture at 37°C, viability of the cell cultures was assessed by dye uptake analysis. The latter was accomplished by decanting the medium from the plates and staining the cells for 10 min with Crystal

Violet (0.5% Crystal Violet, 8% (v/v) formaldehyde (40%), 0.17% NaCl, 22.3% (v/v) ethanol). After staining, the cells were extensively washed with tap water and the dye was dissolved in 100 μ l/well of 33% acetic acid. The plates were read spectrophotometrically at 577 nm with a Titertek multiscan MCC340 ELISA Reader (Flow Laboratories, McLean, VA). Sometimes, the cytotoxic assay was performed in the absence of actinomycin D using an identical experimental set-up but a longer incubation period (48 instead of 18 hours). The latter assay is less sensitive but measures the intrinsic cytotoxic activity of the TNF- α protein.

6.2. In vitro trypanocidal assay

Two times 10^6 /ml purified bloodstream forms of Trypanosoma brucei brucei or T. brucei rhodesiense isolated 1 day before the first *in vivo* maximum peak is reached, were incubated for 5 h in phosphate buffered saline (PBS), 1% glucose, 1% normal mouse serum (incubation medium) at 37°C with various concentrations of recombinant mTNF- α , recombinant hTNF- α or the TNF- α -derived tip peptides, all in the absence or the presence of anti-tip peptide polyclonal or monoclonal antibodies. After 5 h of incubation, the number of live (= moving) parasites was assessed (counting chamber) and compared the control wells in which only incubation medium was added to the trypanosomes. The spontaneous mortality in the control wells after 5 h was always lower than 10%. All treatments were done in quadruplicate and all reported *in vitro* trypanolysis experiments were repeated at least 3 times.

7. In vivo assay to evaluate the tumoricidal effect of human and mouse TNF- α

7.1. Anti-tumor effect of hTNF- α on subcutaneous HT-29 tumors in nude mice.

To test the anti-tumor effect of wild type and tip-mutated or -deleted hTNF- α , 5×10^6 HT-29 human adenocarcinoma (ATCC HTB38) were subcutaneously (s.c.) injected in nude mice (Iffa

Credo, France). The treatment comprises daily perilesional injections for 6 days per week, followed by 1 day without a treatment this for a period of 1 month. Perilesional is a subcutaneous injection near the site of the tumor but outside the tumor nodule. Per mouse, 5 μ g of human recombinant TNF- α was injected. Tumor volume was estimated every 3 or 4 days by measuring the larger (a) and the smaller (b) diameter and calculating the $a \times b^2 \times 0.4$ as known in the art.

7.2. The B16Bl6 melanoma model to test the anti-tumor activity of wild type and tip-mutated or tip-deleted mTNF- α .

5.10⁶ B16Bl6 melanoma cells (Hart and Fidler, 1980) are injected subcutaneously (s.c.) in the back of C57/black mouse (Iffa Credo, France). Between days 8 and 10, a subcutaneous tumor nodule appears at the inoculum site in all animals. Treatment started at day 10 for 10 days and consisted of a daily perilesional injection of 5 μ g mTNF- α /mouse. The tumor volume was estimated as mentioned above.

8. In vivo assays to evaluate the toxic effect or septic shock effect of recombinant hTNF- α or mTNF- α

8.1. Induction of the LPS-induced septic shock syndrome in mice

Intraperitoneal (i.p.) injection of 500 μ g of LPS/mouse causes death within 48 hours. Protection against septic shock syndrome was assayed by injecting 10-weeks old Balb/C mice (10 mice/group) with 100 μ g of the protein G-purified rabbit-anti-mTNF- α -tip peptide polyclonal antibodies in 200 μ l PBS, or with an equal amount of control rabbit polyclonal antibodies or with 200 μ l PBS, 2 h before injection of 500 μ g of LPS/mouse. The number of days of postinjection survival was then assessed.

8.2. Induction of the TNF- α -induced lethal shock

Intravenous (i.v.) injection of 10-week-old C57BL/6 mice

with 10 μ g/mouse of mTNF- α causes death within 48 hours.

Ten weeks old C57BL/6 mice (5 to 6/group) were injected with different doses of wild type or mutant mouse TNF- α and the postinjection survival time was then assessed.

9. Statistics

All data were analyzed using the Mann-Whitney U test.

10. Biotinylated peptide synthesis

All amino acid sequences are given in the conventional and universally accepted three-letter or one-letter code.

The peptide sequences are given left to right which, by convention, is the direction from the amino terminus to the carboxyterminus.

A number of unconventional codes are also used to represent chemical groups or modifications and are defined as follows:

Ac = acetyl group

Bio = D-biotinyl group

Fmoc = 9-fluorenylmethoxycarbonyl group

tBoc = tertiary butyloxycarbonyl group

All of the peptides described were synthesized on TentaGel S-RAM (Rapp Polymere, Tübingen, Germany), a polystyrene-polyoxyethylene graft copolymer functionalized with the acid-labile linker 4-(α -Fmoc-amino-2',4'-dimethoxybenzyl) phenoxyacetic acid (Rink et al., 1987) in order to generate peptide carboxy-terminal amides upon cleavage. t-Butyl-based side chain protection and Fmoc- α -amino-protection was used. The sulfhydryl group of cysteine was protected with a trityl group. Couplings were carried out using preformed O-pentafluorophenyl esters. Biotin was coupled using TBTU as the activating agent in the presence of two equivalents of the base N-methylmorpholine. All syntheses were carried out on a Milligen 9050 PepSynthesizer (Novato, CA) using continuous flow procedures. Following cleavage with trifluoroacetic acid in the presence of scavengers and

extraction with diethylether, all peptides were analyzed by C18-reverse phase chromatography.

11. ELISA assay to quantify wild type and tip-mutated or tip-deleted mouse TNF- α protein

An ELISA assay to measure the protein concentration of the wild type and tip-deleted or tip-mutated mouse TNF- α protein was developed. Thereto, the microtiter plates were coated overnight at room temperature with 100 μ l per well of 2.5 μ g/ml of rabbit anti-mouse TNF- α (Innogenetics, Belgium) in 10 mM Tris, 10 mM NaCl, 10 mM NaN₃. Subsequently, the plates were washed once with washing buffer (PBS, 0.05 % Tween-20) and the remaining binding sites were blocked for at least one hour at 37 °C with 300 μ l per well of PBS buffer supplemented with 1 % BSA and 0.1 % NaN₃. Dilutions of sample or standard mTNF- α (0.01 to 10 ng/ml mTNF- α), made in 100 μ l of PBS supplemented with 1 % BSA, 1 % rabbit serum, and 0.1 % mertiolate are then added to each well and incubated for 2 hours at 37 °C. Thereafter, the plates are washed 4 times with washing buffer and 100 μ l/well of biotinylated rabbit anti-mouse TNF- α which has been diluted with sample diluent to a concentration of 0.125 μ g/ml is added for another 2 hours at 37 °C. Subsequently, the plates are washed four times with washing buffer wherafter 100 μ l of straptavidin-horseradish peroxidase (Jackson ImmunoResearch Labs), which has been diluted in sample diluent to a concentration of 100 ng/ml is added and incubated for 30 minutes at 37 °C. To each well, 100 μ l of TMB solution (8.7 g/l tetramethyl benzidine in DMSO) which has been diluted 100 times in substrate buffer (solution A: 0.1 M Na₂HP₄.2H₂O; solution B: 0.1 M citric acid; solution C: 3 % H₂O₂. Mix 149 parts of solution A with 100 parts of solution B, and add 2 ml of solution C per 1 liter mix) is added and incubated for 30 minutes at room temperature. The reaction is stopped by adding 100 μ l per well of 2 N H₂SO₄ wherafter the optical density is read at dual wavelength (450/595 nm).

EXAMPLE 1: Generation of recombinant wild-type and tip-mutated or -deleted mTNF- α protein in *E. coli*

A schematic overview of the amino acid sequence spanning the region of amino acids 105 to 110 of mTNF- α and all constructed mTNF- α muteins is given in Table II.

Table II

Positions	105	106	107	108	109	110
mTNF- α	Thr	Pro	Glu	Gly	Ala	Glu
T105A mTNF	Ala	Pro	Glu	Gly	Ala	Glu
P106A mTNF	Thr	Ala	Glu	Gly	Ala	Glu
E107A mTNF	Thr	Pro	Ala	Gly	Ala	Glu
E110A mTNF	Thr	Pro	Glu	Gly	Ala	Ala
E107A / E110A mTNF	Thr	Pro	Ala	Gly	Ala	Ala
T105A / E110A mTNF	Ala	Pro	Glu	Gly	Ala	Ala
T105A / E107A mTNF	Ala	Pro	Ala	Gly	Ala	Glu
T105A / E107A / E110A mTNF	Ala	Pro	Ala	Gly	Ala	Ala
del TPEGAE mTNF	-	-	-	-	-	-

1. Construction of pIG2mTNF

A 701 bp TaqI/XbaI fragment, containing the mTNF- α coding sequence except the signal sequence and the first 21 bp of the mature sequence, was isolated from pBSK(+)mTNF. This pBSK(+)mTNF vector contains the full-size mTNF- α cDNA as been published by Fransen et al, (1985).

This fragment was subsequently ligated to the following synthetic DNA sequence :

5'-CATGCTCAGATCAAGTAGTCAAATT-3' (SEQ ID NO 3)
 3'-GTACGAGTCTAGTTCATCAGTTTTAAGC-5' (SEQ ID NO 4)
 ^L startcodon mature mTNF- α

This new 727 bp sequence was then ligated into the SspI/XbaI opened vector pIG2 (SEQ ID NO 2), resulting in the vector pIG2mTNF.

pIG2 is a versatile expression vector for *E.coli*. It contains the temperature-inducible leftward promotor of phage lambda, a synthetic ribosome binding site, a multilinker sequence, the rrnBT1T2 transcriptional terminator and a tetracycline resistance gene (Fig. 1 and 2)

Transformation and subsequent induction of pIG2mTNF to *E.coli* host MC1061(pAcI) resulted in an expression level of $\pm 10\%$ of total cellular protein (data not shown).

2. Construction of mTNF- α Thr105 mutant pIG2mTNFT105A


The mTNF- α Thr105 mutant, wherein the Thr105 was replaced by an Ala residue, was generated by introducing the synthetic oligonucleotide primers 5'- CAAGGACGCTCCGGAGGGGGCTGAGCT-3' and 5'- CAGCCCCCTCCGGAGCGTC-3' in the StyI - SacI digested wild type (wt) mTNF- α , thereby replacing the Thr105 by an Ala residue and creating an unique BstEI control restriction site in the mutated TNF insert, which is not present in the wt TNF- α sequence.

Thereto pIG2mTNF DNA (13 μ l = ± 8 μ g) was cut with BamHI (40 Units, 10 Units/ μ l, Boehringer Mannheim) in a total volume of 80 μ l buffer B (Boehringer Mannheim) for 3 hours at 37°C. The digested DNA was purified via the GeneClean method (Bio101, La Jolla) and resuspended in 68 μ l of sterile doubledistilled water. To this volume 8 μ l of 10 times concentrated buffer H (Boehringer Mannheim) and 40 Units StyI (Boehringer Mannheim, 10 Units/ μ l) were added and again incubated for 3 hours at 37°C. A 911 bp BamHI/StyI fragment was isolated from a 1.2% agarose gel via the GeneClean method and resuspended in 20 μ l sterile doubledistilled water. This material is called fragment 1.

To generate the second fragment, pIG2mTNF DNA (8 μ l = ± 5 μ g) was cut with BamHI (40 U, 10 U/ μ l, Boehringer Mannheim) in a total volume of 80 μ l buffer B (Boehringer Mannheim) for 3 hours at 37°C. The digested DNA was purified via the GeneClean method (Bio101, La Jolla) and resuspended in 68 μ l of sterile

doubledistilled water. To this volume 8 μ l of 10 times concentrated buffer A (Boehringer Mannheim) and 40 Units SacI (Boehringer Mannheim, 10U/ μ l) were added and again incubated for 3 hours at 37°C. A 3071 bp SacI/BamHII fragment was isolated from a 1.2% agarose gel via the gene clean method and resuspended in 40 μ l sterile doubledistilled water. This material is called fragment 2.

To generate the mTNF- α Thr105 mutant pIG2mTNFT105A, 400 pmole (100 pmole/ μ l) of each of the synthetic oligos in a total volume of 10 μ l were heated for 3' at 95°C, 10' at 65°C and were subsequently allowed to reach roomtemperature. The annealed oligos were subsequently placed in ice.

5'-CAAGGACGCTCCGGAGGGGGCTGAGCT-3'	27-mer	(SEQ ID NO 5)
3'-CTGCGAGGCCTCCCCGAC-5'	19-mer	(SEQ ID NO 6)
<div style="text-align: center;">  BspEI </div>		

Fragment 1 (5 μ l), fragment 2 (1.5 μ l) and the annealed oligos (10 μ l) were mixed in a total volume of 19 μ l ligation buffer (Boehringer Mannheim), 1 Units of T4 ligase (Boehringer Mannheim, 1 Unit/ μ l) was added and the mixture was incubated for 3 hours at 15°C. The ligation mixture was transformed to DH1(lambda) competent cells, plated out on Tetracyclin containing LB plates and grown overnight at 37°C. Plasmid DNA was prepared from selected colonies and analysed via restriction digestion (BspEI, BamHI, StyI and SacI). A via restriction analysis confirmed vector pIG2mTNFT105A was transformed to MC1061 (pAcI). This vector/host combination was further used for prokaryotic expression of the mTNFT105A mutein.

3. Construction of mTNF- α Pro106 mutant pIG2mTNFP106A

In this construct the Pro106 of mTNF- α is changed to an Ala residue. In the course of the construction an extra NarI was created in the mTNF coding sequence, which is not present in the wt mTNF sequence.

Thereto pIG2mTNF DNA (13 μ l = \pm 8 μ g) was cut with BamHI (40 Units, 10 Units/ μ l, Boehringer Mannheim) in a total volume of 80

μ l buffer B (Boehringer Mannheim) for 3 hours at 37°C.

The digested DNA was purified via the Geneclean method (Bio101, La Jolla) and resuspended in 68 μ l of sterile doubledistilled water. To this volume 8 μ l of 10 times concentrated buffer H (Boehringer Mannheim) and 40 Units StyI (Boehringer Mannheim, 10U/ μ l) were added and again incubated for 3 hours at 37°C.

A 911 bp BamHI/StyI fragment was isolated from a 1.2% agarose gel via the Geneclean method and resuspended in 20 μ l sterile doubledistilled water. This material was called fragment 3.

To generate the second fragment, pIG2mTNF DNA (8 μ l = \pm 5 μ g) was cut with BamHI (40 Units, 10 Units/ μ l, Boehringer Mannheim) in a total volume of 80 μ l buffer B (Boehringer Mannheim) for 3 hours at 37°C. The digested DNA was purified via the Geneclean method (Bio101, La Jolla) and resuspended in 68 μ l of sterile doubledistilled water. To this volume 8 μ l of 10 times concentrated buffer A (Boehringer Mannheim) and 40 Units SacI (Boehringer Mannheim, 10U/ μ l) were added and again incubated for 3 hours at 37°C.

A 3071 bp SacI/BamHII fragment was isolated from a 1.2% agarose gel via the Geneclean method and resuspended in 40 μ l sterile doubledistilled water. This material was called fragment 4.

To generate the mTNF- α Pro106 mutant pIG2mTNFP106A, 400 pmole (100 pmole/ μ l) of each of the synthetic oligos in a total volume of 10 μ l were heated for 3' at 95°C, 10' at 65°C and were subsequently allowed to reach room temperature. The annealed oligos were subsequently placed on ice.

5'-CAAGGACACCGCTGAGGGCGCCGAGCT-3'	27-mer	(SEQ ID NO 7)
3'-CTGTGGCGACTCCCGCGGC-5'	19-mer	(SEQ ID NO 8)
<div style="border-top: 1px solid black; width: 100px; margin: 0 auto;"></div> NarI		

Fragment 3 (5 μ l), fragment 4 (1.5 μ l), and the annealed oligos (10 μ l) were mixed in a total volume of 19 μ l ligation buffer (Boehringer Mannheim). One Unit of T4 ligase (Boehringer Mannheim, 1 U/ μ l) was added and the mixture was incubated for 3 hours at 15°C. The ligation mixture was transformed to

DH1(lambda) competent cells, plated out on tetracyclin containing LB plates and incubated at 37°C. Plasmid DNA was prepared from selected colonies and analyzed via restriction digestion (NarI, BamHI, StyI and SacI). A vector confirmed via restriction analysis (pIG2mTNFP106A) was transformed to MC1061 (pAcI). This vector/host combination was further used for expression of the mTNFP106A mutein.

4. Construction of other tip mutated mTNF- α

4.1. Construction of pIG2mTNFE107A

In this construct, the Glu107 residue of mTNF was replaced with an Ala residue. In the course of the construction, a unique PstI restriction site was created in the mTNF coding sequence, which is not present in the wt mTNF sequence.

Construction of the vector was similar to the previously constructed mutants with the following differences:

fragment 1 = 911 bp BamHI/StyI fragment from pIG2mTNF

fragment 2 = 3071 bp SacI/BamHI fragment from pIG2mTNF

oligos

```

      K D T P A G A E
5'-CAAGGACACCCCTGCAGGCGCTGAGCT-3' 27-mer (SEQ ID NO 9)
      3'-CTGTGGGGACGTCCGCGAC-5'    19-mer (SEQ ID NO 10)
          └──────────┘

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PstI

4.2. Construction of pIG2mTNFE110A mutant

In this construct, the Glu110 residue of mTNF was replaced with an Ala residue. In the course of the construction, a unique NarI restriction site was created in the mTNF coding sequence, which is not present in the wt mTNF sequence. Moreover, the unique SacI restriction site present in the wt mTNF sequence was destroyed.

Construction of the vector was similar to the previous constructed mutants with the following differences :

fragment 1 = 911 bp BamHI/StyI fragment from pIG2mTNF

fragment 2 = 3071 bp SacI blunt/BamHI fragment from pIG2mTNF

Blunting of the SacI site was done by cutting the pIG2mTNF vector with SacI, purifying the linear DNA via the gene clean

method (resuspended in 20 μ l distilled water).

DNA in 20 μ l water

- + 3 μ l of a 500 mM Tris-Cl pH 7.4, 5 mM MgCl₂, 2 mM β -Me EtOH solution
- + 1 μ l dXTP solution 5 mM each
- + 2 μ l T4 polymerase (1U/ μ l)
- + 4 μ l doubledistilled water

The mixture specified above was incubated for 60 min at 37°C followed by the purification of the blunted DNA via the gene clean procedure and digestion of the DNA with BamHI.

oligos

K D T P E G A A
 5'-CAAGGACACCCCTGAGGGCGCCGCCCT-3' 27-mer (SEQ ID NO 11)
 3'-CTGTGGGGACTCCCGCGGCGGGA-5' 23-mer (SEQ ID NO 12)
└──────────┘
 NarI

4.3. Construction of pIG2mTNFE107A/E110A

In this construct, the Glu107 and Glu110 residues of mTNF were replaced with Ala residues. In the course of the construction unique NarI and PstI restriction sites were created in the mTNF coding sequence, which were not present in the wt mTNF sequence. Moreover, the unique SacI restriction site present in the wt mTNF sequence was destroyed.

Construction of the vector was similar to the previous constructed mutants with the following differences :

fragment 1 = 911 bp BamHI/StyI fragment from pIG2mTNF

fragment 2 = 3071 bp SacI blunt/BamHI fragment from pIG2mTNF

oligos

K D T P A G A A
 5'-CAAGGACACCCCTGCAGGCGCCGCCCT-3' 27-mer (SEQ ID NO 13)
 3'-CTGTGGGGACGTCCGCGGCGGGA-5' 23-mer (SEQ ID NO 14)
└──────────┘
└────────┘ NarI
 PstI

4.4. Construction of pIG2mTNFT105A/E110A

In this construct, the Thr105 and Glu110 residues of mTNF were replaced with Ala residues. In the course of the construction unique NarI and BspEI restriction sites were created in the mTNF coding sequence, which were not present in the wt mTNF sequence. Moreover, the unique SacI restriction site present in the wt mTNF sequence was destroyed.

Construction of the vector was similar to the previous constructed mutants with the following differences :

fragment 1 = 911 bp BamHI/StyI fragment from pIG2mTNF

fragment 2 = 3071 bp SacI blunt/BamHI fragment from pIG2mTNF

oligos

```

      K D A P E G A A
5'-CAAGGACGCTCCGGAGGGCGCCGCT-3' 27-mer (SEQ ID NO 15)
3'-CTGCGAGGCCTCCGCGGCGGGA-5' 23-mer (SEQ ID NO 16)
      [       ] [       ]
      BspEI  NarI

```

4.5. Construction of pIG2mTNFT105A/E107A

In this construct, the Thr105 and Glu107 residues of mTNF were replaced with Ala residues. In the course of the construction unique NciI and BsgI restriction sites were created in the mTNF coding sequence, which were not present in the wt mTNF sequence.

Construction of the vector was similar to the previous constructed mutants with the following differences :

fragment 1 = 911 bp BamHI/StyI fragment from pIG2mTNF

fragment 2 = 3071 bp SacI/BamHI fragment from pIG2mTNF

oligos

```

      K D A P A G A E
5'-CAAGGATGCCCCGGCGGGTGAGAGCT-3' 27-mer (SEQ ID NO 17)
3'-CTACGGGGCCGCCACGTC-5' 19-mer (SEQ ID NO 18)
      [       ] [       ]
      NciI   BsgI

```

4.6. Construction of pIG2mTNFT105A/E107A/E110A

In this construct, the Thr105, Glu107 and Glu110 residues of mTNF were replaced with Ala residues. In the course of the construction, unique EagI and SfiI restriction sites were created in the mTNF coding sequence, which were not present in the wt

mTNF sequence. Moreover, the unique SacI restriction site present in the wt mTNF sequence was destroyed.

Construction of the vector was similar to the previously constructed mutants with the following differences:

fragment 1 = 911 bp BamHI/StyI fragment from pIG2mTNF

fragment 2 = 3071 bp SacI blunt/BamHI fragment from pIG2mTNF

oligos

```

      K D A P A G A A
5' -CAAGGATGCTCCGGCCGGTGC GGCCCT-3' 27-mer (SEQ ID NO 19)
      3' -CTACGAGGCCGGCCACGCCGGA-5' 23-mer (SEQ ID NO 20)
              └──────────┘
              SfiI
              EagI

```

5. Construction of the tip-deleted mTNF- α or pIG2mTNF TPEGAE

In this construct, the TPEGAE coding sequence is deleted from the mTNF gene. In the course of the construction a unique NcoI restriction site was created in the mTNF coding sequence, which is not present in the wt mTNF sequence.

Construction of the vector was similar to the previously constructed mutants with the following differences :

fragment 1 = 911 bp BamHI/StyI fragment from pIG2mTNF

fragment 2 = 3046 bp BstXI/BamHI fragment from pIG2mTNF

oligos

```

      K D L K P W Y E P I Y
5' -CAAGGACCTCAAACCATGGTATGAGCCCATATAC-3' 34-mer (SEQ ID NO 21)
      3' -CTGGAGTTTGGTACCATACTCGGGTA-5' 26-mer (SEQ ID NO 22)
              └──────────┘
              NcoI

```

The construction of additional tip-region deleted mTNF- α mutants also lies within the object of the present invention. These additional mutants are to be constructed using techniques known in the art as described above.

6. E. coli expression of wild type and tip-mutated or deleted mTNF- α .

Batch fermentations were conducted in LB medium (0.5% yeast extract, 1% bactotryptone, 0.5% NaCl), at a 15-liter scale, with a minimum dissolved oxygen content of 50%. pH was maintained

constant during the whole fermentation at a value of 6.9. Foaming problems were avoided by adding 0.013% polypropylene glycol P2000.

Precultures of the different vector/host combinations were grown overnight in LB medium supplemented with tetracycline 0,001% for 18 hours at 28°C. After inoculation of the medium with a final OD₆₅₀ of 0.05, the bacteria were subsequently grown for 4 generations at 28°C, and for 4 hours at 42°C. At the end of the fermentation, the cells were separated from the medium by centrifugation, suspended in lysis buffer (10 mM KCl, 10 mM Tris-HCl, pH 6.8, 5 mM EDTA) and stored at -70°C.

7. Purification of recombinant wild-type and tip-mutated or deleted mTNF- α .

7.1 Purification of wild type mouse TNF- α

To the bacterial cell suspension, aminocaproic acid, PMSF and DTT are added to a final concentration of respectively 25 mM, 1 mM and 1 mM before lysing by French Press (14,000 psi, AMINCO, SLM Instruments, Urbana, USA). Subsequently, the suspension is centrifugated for 20 minutes at 16,500 rpm at 4°C using a JA-20 rotor (Beckman). Wild-type mTNF- α is found in the supernatant fraction as assessed by SDS-polyacrylamide gelelectrophoresis (SDS-PAGE). Some of the TNF- α tip muteins were retrieved in the soluble and insoluble fraction. The described procedure is directed to the recovery of the mTNF- α material out of the soluble fraction. No attempts were made to purify and renature mTNF from the insoluble fraction. The nucleic acids in the supernatant were precipitated at pH 7.0 by the addition of polyethylene-imine to a final concentration of 0.4% followed by centrifugation by a JA-20 rotor for 20 minutes at 16,500 rpm at 4°C. mTNF- α or mutein protein were then enriched by a differential ammoniumsulphate precipitation whereby a 40% step is followed by a second step at 65% saturation. mTNF- α or muteins remained in the 65% ammonium sulphate pellet.

The pellet was resuspended in 25 mM Tris-HCl pH 7.2 and the conductivity of the solution was adjusted to 10 mS above the

conductivity of the Phenyl sepharose column startbuffer (25 mM TrisHCl pH 6.9, 25% (NH₄)₂SO₄). The material was then loaded at 0.5 cm/minute on the Phenyl Sepharose 4B matrix (Pharmacia) and washed with 8 column volumes of starting buffer. Proteins bound to the matrix were eluted with a linear salt gradient of 0% to 60% buffer B (20 mM ethanolamine, 10% ethylene glycol pH 9.0) over 6 column volumes.

The flow through fractions containing the mTNF- α protein were pooled and dialysed overnight against 25 mM Tris-HCl pH 7.2. After centrifugation, the material was loaded on Q Sepharose FF (Pharmacia) equilibrated against 25 mM Tris-HCl pH 7.2 at 1 cm/minute. The column was washed with 4 column volumes of starting buffer and eluted with a linear salt gradient from 0 mM to 300 mM NaCl in the same buffer followed by stepwise elution with 2 column volumes at 1 mM NaCl. Recombinant mTNF- α eluted at 300 mM NaCl.

By this procedure 98% pure mTNF- α could be obtained with a molecular mass of 17 kDa, as defined by SDS-PAGE under reducing conditions, and a specific activity of $\pm 2 \times 10^8$ U/mg as assessed by bioactivity on L929 cells.

7.2. Purification of P106A mTNF α mutein

Cell lysis, clearing and polyethylene imine treatment were performed as described in 7.1. In contrast to the physico chemical behaviour of wt mTNF, the P106A mutein was recovered in the PEI pellet. Therefore, the pellet was extracted with 20 mM Tris-HCl, pH-7.0, 0.5 M NaCl (200 ml/pellet) and cleared by centrifugation (JA 20 rotor, 16 500 rpm, 20 min, 4°C). The purification process ((NH₄)₂SO₄ fractionation and chromatography steps on Phenyl Sepharose 4B and Q Sepharose FF) were identical to those described in the section 7.1 as for wt mTNF- α . Over 95% pure P106A mTNF- α was obtained by this procedure with a specific activity of $\pm 1.81 \times 10^8$ U/mg as assessed by bioassay on L-929 cells in the presence of Actinomycin D.

7.3. Purification of the T105A mTNF α mutein

Cell lysis, clearing and PEI treatment of the supernatants were performed as described in for wild type TNF- α . As for the P106A mutein, the T105A mTNF- α was recovered in the PEI pellet. Extraction and clearing were therefore performed as already described in section 7.2 for the P106A mutein.

The conductivity of the supernatant was adjusted to 10 mS above this of the equilibration buffer of the Phenyl Sepharose 4B column. The chromatographical conditions were identical to those described in section 7.1, except that an equivalent of a 5 liter cell culture was loaded on a 40 ml column. The T105A mTNF- α mutein was recovered in the first part of the gradient (about 0.9 M \rightarrow 0,5 M (NH₄)₂SO₄). The pools of the 3 runs on Phenyl Sepharose 4B were mixed and after adjustment of the pH and the conductivity, the total pool was loaded on a 16 ml Phenyl Q Sepharose FF column. Chromatography conditions (flow rate (cm/h) and slope of the gradient) were kept identical as for these used on the previous Phenyl Sepharose 4B column.

Strongly enriched mTNF- α mutein was found in the flow through and wash as well as in the 100 % B eluate (20 mM ethanol amine, 10% ethylene glycol, pH 9.0). All TNF- α containing fractions were collected and both pools were dialysed separately against 20 mM Tris-HCl, pH 7.2 and loaded on a Q Sepharose (Pharmacia), equilibrated with the same buffer. The chromatographical conditions were identical to those described as for wt mTNF- α (see section 7.1).

SDS PAGE under reducing conditions showed that from the Phenyl Sepharose 4B eluate a preparation of more then 95% pure T105A TNF material could be obtained with a specific activity of $\pm 1 \times 10^8$ U/mg as assessed by bioassay on L929 cells in the presence of Actinomycin D.

7.4 Purification of tip deleted mTNF- α mutein (del mTNF- α)

Cell lysis, PEI treatment of the supernatant as well as the (NH₄)₂SO₄ fractionation were identical as these described for the P106A mTNF- α mutein. The chromatography on Phenyl Sepharose 4B was identical to this described in 7.1 (wt mTNF) except that 7,5

cell culture equivalent was loaded on a 40 ml column (this means 2 runs instead of 4). The conductivity of the non-bound fraction of the phenyl sepharose runs was adjusted to 10 mS above this of the equilibration buffer of the Phenyl Sepharose 4B column and the material was loaded on a 10 ml TSK butyl 650 M column (Merck) (flow rate: 1,5 cm/min; 10 mg protein/ml gel; protein quantification based on absorbance at 280 nm). The buffers for chromatography on TSK butyl 650 M are identical to those used in the chromatography on Phenyl Sepharose 4B. After an extensive wash with equilibration buffer (12 column volumes), bound proteins were eluted by applying a linear gradient over 16 column volumes (100% A - 100% B).

The non-bound protein fraction was dialysed against 25 mM Tris-HCl, pH 9.0 and applied at 1cm/min on a Q Sepharose FF column (Pharmacia) (> 5mg protein/ml gel). Bound proteins were eluted at 1 cm/min by applying a linear salt gradient (0 - 300 mM NaCl), followed by a 300 mM NaCl wash (2 column volumes). SDS-PAGE shows that the del mTNF- α protein was found all over the eluate, but only the fractions eluting between 70-230 mM NaCl were pooled. These fractions were lacking the contaminating E. coli proteins with a molecular weight of about 14000, 15000 and 70000. The pooled fractions were diluted with the 25 mM Tris-HCl, pH 9.0 buffer and incubated for about 4 h at 4°C. Thereafter, the del mTNF- α pool was reloaded on the Q-sepharose FF column but now at a concentration < 300 μ g protein/ml gel (protein quantity based on absorbance at 280 nm). The wash and elution conditions were identical to these described for the first chromatography on Q Sepharose. The tip-deleted mTNF- α protein eluted in 2 well separated peaks with pure del mTNF- α protein in the second peak (elution at about 280 mM NaCl). The material has a specific activity of about 1×10^8 U/mg of protein as assessed by bioassay on L-929 cells in the presence of Actinomycin D.

7.5. Purification of the T105A-E107A mTNF- α double mutant

To the bacterial cell suspension, aminocaproic acid, PMSF and DDT were added to a final concentration of respectively 25

mM, 1 mM and 1 mM before lysing by French Press (14,000 psi, AMINCO, SLM Instruments, Urbana, USA). Subsequently, the suspension was centrifugated for 20 minutes at 16,500 rpm at 4°C using a JA-20 rotor (Beckman). The mTNF- α double mutant could be found back in the supernatant fraction as assessed by SDS-PAGE and therefore only this fraction was used for further purification. The nucleic acids in the supernatant were precipitated by the addition of polyethylene-immine to a final concentration of 0.4% followed by centrifugation for 20 minutes at 16,500 rpm at 4°C. The mTNF- α double mutant precipitated only partially after polyethylene imine treatment and was recovered from the pellet by introducing a washing step with 0.5 ml NaCl pH 7.2. Both fractions were then enriched by a differential amoniumsulphate precipitation whereby a 40% step is followed by a second step at 65% saturation. The mTNF- α mutein remained in the 65% amonium sulphate pellet.

The pellet was resuspended in 25 mM Tris-HCl pH 7.2 and the conductivity of the solution was adjusted to 10 mS above the conductivity of the Phenyl Sepharose 4B column startbuffer (25 mM TrisHCl pH 6.9, 25% (NH₄)₂SO₄). The material was loaded at 0.5 cm/minute on the Phenyl Sepharose 4B matrix (Pharmacia) and washed with 8 column volumes of starting buffer. Proteins bound to the matrix were eluted with a linear salt gradient of 0% to 60% buffer B (20 mM ethanolamine, pH 9.0) over 6 column volumes.

The flow through fractions containing the mTNF- α double mutant protein were pooled and dialysed overnight against 25 mM Tris-HCl pH 7.2. After centrifugation, the material was loaded at 1 cm/minute on Q Sepharose FF (Pharmacia) equilibrated against 25 mM Tris-HCl pH 7.2. The column was washed with 4 column volumes of starting buffer and eluted with a linear salt gradient from 0 mM to 300 mM NaCl in the same buffer followed by stepwise elution with 2 column volumes of 300 mM NaCl and 2 column volumes of 1 mM NaCl. The mTNF- α double mutant eluted at 300 mM NaCl. A further purification was performed on a column of Fractogel TSK butyl 650M (Merck). To the protein solution, 25% (NH₄)₂SO₄ was added and loaded on the column at a flow-rate of

1 cm/minute (3 mg/ml gel). The mTNF- α mutein protein was recovered in the flow through fraction and the proteins bound to the matrix were eluted with 10 column volumes of a linear gradient from 0% to 100%. As a final polishing step the T105A-E107A mTNF protein was purified by gel filtration on a Superdex 75 HR 10/30 column (Pharmacia) whereon it eluted as the second (or largest) protein peak with an apparent weight of 66 kD.

7.6 Purification of the T105A-E107A-E110A mTNF- α triple mutant

To the bacterial cell suspension, aminocaproic acid, PMSF and DDT was added to a final concentration of respectively 25 mM, 1 mM and 1 mM before lysing by French Press (14,000 psi, AMINCO, SLM Instruments, Urbana, USA). Subsequently, the suspension was centrifugated for 20 minutes at 16,500 rpm at 4°C using a JA-20 rotor (Beckman). The mTNF- α mutein could be found back in the supernatant fraction as assessed by SDS-PAGE. The nucleic acids in the supernatant was then precipitated by the addition of polyethylene-imine to a final concentration of 0.4% followed by centrifugation for 20 minutes at 16,500 rpm at 4°C. The mTNF- α triple mutant was then enriched by a differential ammoniumsulphate precipitation whereby a 40% step is followed by a second step at 65% saturation. mTNF- α or muteins remain in the 65% ammonium sulphate pellet.

The pellet was resuspended in 25 mM Tris-HCl pH 7.2 and the conductivity of the solution was adjusted to 10 mS above the conductivity of the Phenyl Sepharose 4B column startbuffer (25 mM Tris-HCl pH 6.9, 25% (NH₄)₂SO₄). The material was loaded at 0.5 cm/minute on the Phenyl Sepharose 4B matrix (Pharmacia) and washed with 8 column volumes of starting buffer. Proteins bound to the matrix were eluted with a linear salt gradient of 0% to 60% buffer B (20 mM ethanolamine, pH 9.0) over 6 column volumes. The fractions containing the mTNF- α triple mutant (this is the flow through and the 20 to 40% eluate fractions) were pooled and loaded on a TSK-C4 650M (Merck) at 1 cm/h (10 mg/ml) and washed with 8 column volumes of Phenyl Sepharose starting buffer. The

proteins bound to the matrix were eluted with a linear gradient of 10 column volumes from 0% B to 100% B (20 mM ethanolamine pH 9.0).

The flow through fractions containing the mTNF mutein protein were pooled and loaded at a flow rate of 1 cm/min (5 mg/ml protein) on a Cu²⁺ IDA Sepharose fast flow column (Pharmacia) equilibrated against 20 mM sodium phosphate, 500 mM NaCl pH 6.8. The protein bounding to the matrix were eluted with a linear gradient from 0% to 70% B (20 mM sodium phosphate, 0,75 M NH₄Cl pH 7.8) during 10 column volumes, one column volume of 70% B and one column volume of 100% B. The protein of interest eluted in the 70% B fraction. The fractions were pooled and dialysed against 25 mM Tris-HCl pH 9,0.

The material was then loaded on a Q Sepharose FF (Pharmacia) column equilibrated against 25 mM Tris-HCl pH 7.2 at 1 cm/minute. The column was washed with 4 column volumes of starting buffer and eluted with a linear salt gradient from 0 mM to 300 mM NaCl in the same buffer followed by stepwise elution with 2 column volumes of 300 mM NaCl. The triple mTNF- α mutant eluted at 250 mM NaCl.

EXAMPLE 2: The trypanocidal region of TNF- α contains a lectin-like activity of the molecule

1. Recombinant mTNF- α and hTNF- α are trypanocidal for T. brucei brucei and T. brucei rhodesiense and the effect is potentiated by cotreatment with LiCl

Incubation of purified bloodstream forms of T. brucei brucei (Fig. 3 A) and T. brucei rhodesiense (Fig. 3 B) for 5 h with rm/hTNF- α results in the mortality of a part of the parasites: 50% of the animals are killed with 5000 pg/ml of rm/hTNF- α on T. brucei brucei and T. brucei rhodesiense. In the same assay, equal concentrations of hTNF- β , mouse interleukin 1 α or mouse interleukin 1 β do not cause any mortality of T. brucei brucei or T. brucei rhodesiense (data not shown). As shown in Fig. 4, this trypanocidal effect of TNF- α is potentiated 100-fold by co-

treating the trypanosomes with 1 $\mu\text{g/ml}$ of LiCl, demonstrating that not only the cytotoxic effect of TNF- α on tumor cells (Beyaert et al., 1989) but also the trypanocidal activity of the molecule is increased by LiCl.

2. The trypanocidal activity of mTNF- α *in vitro* is inhibited by preincubating TNF- α with a trypanosome lysate, whereas preincubation of the mTNF- α with the soluble p55 hTNF- α receptor protein does not block this effect

Preincubation of the mTNF- α during 1h with different concentrations of a lysate from *T. brucei brucei* prepared as described in the materials section potently inhibits the mTNF- α trypanocidal effect (Fig. 5a), whereas the tumoricidal activity of mTNF- α on L929 cells is not affected (Fig. 5b).

On the other hand, preincubation of the mTNF- α during 1 h with 5 $\mu\text{g/ml}$ of the soluble p55 hTNF- α receptor potently inhibits the mTNF- α tumoricidal activity against L929 cells, whereas the trypanocidal effect is hardly influenced (Table III). This result clearly demonstrates that the region responsible for the trypanocidal activity of the TNF- α molecule can be separated from the cellular p55 receptor binding domain on the TNF- α protein.

TABLE III: Inhibitory capacity of soluble p55 hTNF receptor on the mTNF- α tumoricidal and trypanocidal activity

mTNF- α (pg/ml)	Trypanocidal activity (% lysis)			Tumoricidal activity (% lysis)		
	-p55	+p55	Δ	-p55	+p55	Δ
100	30 \pm 2	25 \pm 3	15 \pm 5	40 \pm 2	0 \pm 1	100 \pm 5
1000	45 \pm 5	41 \pm 4	10 \pm 4	92 \pm 3	0 \pm 1	100 \pm 4
10000	60 \pm 4	56 \pm 3	7 \pm 5	98 \pm 4	93 \pm 3	5 \pm 2

Table III: Δ :% inhibition of activity by the addition of the p55 hTNF- α receptor protein

3. The TNF- α lectin-like region is probably involved in the trypanocidal activity of the protein

As shown in Fig. 6A, preincubation of mTNF- α for 1 h with 1 μ g/ml of N,N'-diacetylchitobiose, an oligosaccharide for which TNF- α has been shown to have a lectin-like affinity (Hession et al., 1987; Sherblom et al., 1988), results in a potent inhibition of the mTNF- α trypanocidal effect, whereas the mTNF- α tumoricidal activity as measured on L929 cells is not affected (Fig. 6B.)

Moreover, preincubation of the *T. brucei brucei* trypanosomes with lectins (added at a concentration of 10 μ g/ml) that have a specificity for N,N'-diacetylchitobiose, such as *Urtica dioica* agglutinin and *Triticum vulgaris* agglutinin, also results in an inhibition of the mTNF- α trypanocidal activity, whereas lectins with a different specificity (and added at the same concentration of 10 μ g/ml), such as *Galanthus nivalis* agglutinin and *Griffonia simplicifolia* A4, did not have any effect upon preincubation with the mTNF- α (Table IV). Moreover, none of these lectins had any effect on the tumoricidal activity of TNF- α on L929 cells.

These results strongly suggest that the TNF- α lectin-like region is involved in the trypanocidal, but not in the tumoricidal activity.

TABLE IV: Inhibitory capacity of different lectins on mTNF- α mediated trypanocidal activity against *T. brucei brucei*

mTNF- α (pg/ml)	% inhibition of trypanocidal activity			
	WGA	UDA	GNA	GSI-A4
100	70 \pm 4	80 \pm 2	0 \pm 1	0 \pm 2
1.000	63 \pm 5	63 \pm 3	5 \pm 3	0 \pm 2
10.000	13 \pm 5	29 \pm 5	5 \pm 2	2 \pm 1
100.000	5 \pm 3	3 \pm 2	3 \pm 2	0 \pm 2
1.000.000	7 \pm 5	2 \pm 1	2 \pm 1	0 \pm 1

WGA : *Triticum vulgaris* agglutinin

UDA : Urtica dioica agglutinin

GNA : Galanthus nivalis agglutinin

GSI-A4 : Griffonia simplicifolia A4

All lectins were added at the concentration of 10 µg/ml

Example 3: The tip region of mouse and human TNF- α contains the trypanocidal activity and lectin-like activities of the molecule and is involved in the TNF-induced lethal shock

1. Design of the TNF- α -derived peptides

The biotinylated tip peptides, inspired on the sequence spanning amino acid positions 100 to 116 of hTNF- α (Pennica et al., 1984) and mTNF- α (Fransen et al., 1985) were synthesized as indicated in the materials and methods section. Subsequently, the peptides were analyzed using a C18 reversed phase HPLC column. Analysis of the three-dimensional structure of the TNF- α molecule upon consulting the Brookhaven data base demonstrated that the identified epitope is located at the tip of the molecule. This location is interesting considering the fact that TNF- α was shown to be active in a membrane-bound form (Kriegler et al., 1988). The following substitutions in both m/hTNF- α tip regions were made: P(100) by C, C(101) by G, and E(116) by C. It should be noted that the wild-type sequence of TNF- α contains a Cys residue at location 101. In order to prevent inopportune closing of the loop with this Cys(101), resulting in an unwanted conformation, the Cys 101 residue was mutated to a Gly. It has been demonstrated that this mutation did not affect the cytotoxic activity of native TNF- α , indicating that this amino acid is not involved in the TNF-receptor binding (Arakawa et al., 1990). Furthermore, native TNF- α as well as the tip peptide exert a trypanocidal activity as assayed by trypanosome survival in the in vitro assay. An overview of all the peptides synthesized is given in Table V below.

TABLE V: Overview of synthetic TNF- α tip region peptides.

mTNF α -tip	: Bio-GG-CGPKDTPEGAE LKPWYC	(SEQ ID NO 24)
hTNF α -tip	: Bio-GG-CGQRETPEGAE AKPWYC	(SEQ ID NO 25)
subpep 1	: Bio-GG-C----TPEGAE-----C	(SEQ ID NO 26)
subpep 2	: Bio-GG-----EGAE LKPWY-	(SEQ ID NO 27)
subpep 3	: Bio-GG-----TPE-----	(SEQ ID NO 28)
subpep 4	: Bio-GG-c----tpeGae-----c	(SEQ ID NO 29)
mutpep 1	: Bio-GG-C----APEGAE-----C	(SEQ ID NO 30)
mutpep 2	: Bio-GG-C----TAEGAE-----C	(SEQ ID NO 31)
mutpep 3	: Bio-GG-C----TPAGAE-----C	(SEQ ID NO 32)
mutpep 4	: Bio-GG-C----TPEAAE-----C	(SEQ ID NO 33)
mutpep 5	: Bio-GG-C----TPEGAA-----C	(SEQ ID NO 34)

The sequences are given in the one letter amino acid code. The amino acids in the L or D configuration are anotated in the one letter code in capitals or in small letters, respectively. The annotation Bio-GG- denotes Biotin-Gly-Gly.

2. The synthetic mTNF- α tip peptide is directly trypanocidal in vitro, an effect that can be inhibited by preincubation of the peptide with N,N'-diacetylchitobiose

To prove that the tip region of mTNF- α is directly implicated in its trypanocidal and its lectin-like effect, experiments were designed to test (i) whether the synthetic mTNF- α -tip peptide has any trypanocidal activity in vitro and (ii) if so, whether this activity can be inhibited by preincubating the peptide with N,N'-diacetylchitobiose. As shown in Fig. 7, the mTNF- α -tip peptide indeed shows a trypanocidal activity against bloodstream forms of T. brucei brucei, whereas it exerts no tumoricidal activity on L929 cells when tested in a similar dose response curve (data not shown). The hTNF- α -tip peptide shows similar results (Fig. 7). In the in vitro trypanocidal assay, h/mTNF- α has a specific activity of 2×10^5 U/mg of protein, while the peptide exerts its activity with an

average specificity of 4×10^2 - 4×10^3 U/mg of peptide. Interestingly, the trypanocidal activity of the peptide can also be inhibited by preincubating it for 1 h with 1 μ g/ml of N,N'-diacetylchitobiose (Fig. 7), demonstrating that the tip region of h/mTNF- α (from amino acid position 100 to 116) is involved in its trypanocidal and its lectin-like activity.

3. The short tip peptide, Thr Pro Glu Gly Ala Glu (SEQ ID NO 23), contains the trypanocidal activity of TNF- α and is linked with the described lectin-like activity of the protein since it can be inhibited by preincubation with N,N'-diacetylchitobiose

The fact that both human and mouse TNF- α peptides exert *in vitro* trypanocidal activity with no significant differences in specific activity also implies that the amino acids which are common to the human and the mouse TNF- α tip region can be involved in the trypanocidal activity. This hypothesis was further investigated by testing the trypanocidal activity of two subpeptides of the tip region containing sequences similar to both species. The subpeptide 1, which consists of the positions T(105), P(106), E(107), G(108), A(109) and E(110) which are identical in m/hTNF- α still had full trypanocidal activity (Fig. 8), whereas subpeptide 2 which encompasses the positions E(107) to Y(115) of mTNF- α (with only L(111) different from hTNF- α) showed strongly reduced trypanocidal activity (Fig. 9). These results strongly suggest that the trypanocidal and, thus, perhaps also the lectin-like activity of TNF- α , is at least partially mediated by amino acid position T(105) to E(110), in which especially positions T(105) and/or P(106) seem(s) to be of the utmost importance. The trypanocidal activity of the different mutant peptides 1, 2, 3, 4 and 5 of h/mTNF- α was measured, wherein positions 105, 106, 107, 108 or 110 of the TPEGAE peptide were replaced consecutively by an A. The mutant peptides in which the T(105) or E(107) or E(110) was replaced by an A lost their trypanocidal activity, while the two other mutants were fully or even more active (Fig. 8 and 9).

Comparison of the trypanocidal activity of the fully active

TPEGAE peptide with the synthetic oligopeptide tpeGae wherein the L amino acids were replaced by D residues demonstrated the stereospecificity of the TNF- α tip peptide trypanosome interaction since the latter peptide had lost its activity (Fig. 8).

EXAMPLE 4 : Antibodies raised against the tip region of mouse and human TNF- α inhibit the trypanocidal activity and the septic shock effects but not the tumoricidal activity of TNF- α .

1. Generation of rabbit anti-tip mTNF- α and hTNF- α polyclonal antibodies

Polyclonal antibodies against the mTNF- α and hTNF- α -derived tip peptides were produced as follows: On day 0, rabbits were injected subcutaneously with 50 μ g of a complex existing of 7 μ g of the biotinylated tip peptide and 43 μ g of avidin (Neutralite, Eurogentec, Belgium) dissolved in complete Freund's adjuvant (total volume: 1 ml) (Difco Laboratories, Detroit, MI, USA). On day 21, rabbits were injected subcutaneously with 50 μ g of the same complex dissolved in incomplete Freund's adjuvant (total volume: 1 ml). On day 42, the rabbits were injected subcutaneously with 100 μ g of the avidin-tip peptide complex in 1 ml of PBS. On day 52, the rabbits were bled, and the sera which reacted positively in ELISA with both the synthetic h/mTNF- α tip peptide bound to streptavidin and with the native h/mTNF- α molecule, were purified on Protein G Sepharose CL4B (Sigma, St. Louis, MO, USA) essentially as proposed by the supplier. The IgG concentration was assessed by the Bradford assay.

2. Generation of anti-tip mTNF- α monoclonal antibodies

2.1. Immunization

Balb/c mice were immunized with 100 μ g of avidine-biotine-TNF- α tip peptide conjugates emulsified in complete Freund's adjuvant. Three weeks later the same animals were challenged with 400 μ g of avidin. Biotin-TNF- α tip peptide conjugates emulsified in incomplete Freund's adjuvant. Another three weeks later, the

mice were boosted with 10 μ g of avidin-biotin-TNF- α tip peptide conjugates dissolved in PBS. Three days later the spleen cells were fused to generate monoclonal antibodies.

2.2. Fusion and screening

Approximately 10^8 spleen cells of immunized Balb/c mice were fused with the universal B cell fusion partner NSO via the PEG-fusion protocol (Galfré and Milstein, 1981). Positive hybridomas were identified via an ELISA screening on ELISA plates coated with TNF- α . The thus obtained anti-TNF- α tip monoclonal antibodies did not neutralize the tumoricidal activity of TNF- α .

3. Differential inhibition of the trypanocidal and the tumoricidal activities of mTNF- α by monospecific polyclonal anti-mTNF- α tip peptide antibodies or monoclonal mTNF- α anti-tip antibodies.

Preincubation of mTNF- α for 1 h at room temperature with 10 μ g/ml of protein G-sepharose-purified rabbit-anti-rmTNF- α tip peptide polyclonal antibodies or with a mouse anti-rmTNF- α tip peptide monoclonal antibody results in a strong inhibition of the mTNF- α -mediated trypanocidal effect (Fig. 10), whereas only a weak inhibition of the mTNF- α -mediated tumoricidal effect on L929 cells was detected (data not shown).

The results stress once more that this region of the TNF- α molecule is directly involved in the trypanocidal and in the lectin-like activity of TNF- α .

4. Anti-mTNF- α tip polyclonal antibodies can inhibit LPS-induced septic shock *in vivo*

Sherblom et al. (1988) have shown that TNF- α has a lectin-like affinity for the glycoprotein uromodulin. Interestingly, this glycoprotein is able to potently inhibit the LPS-induced shock *in vivo*, whereas it does not block the tumoricidal activity of TNF- α on L929 cells *in vitro*. The authors suggested therefore that the lectin-like region of TNF- α is implicated in the toxic effects of TNF- α in septic shock, but not

in its tumoricidal activity on L929 cells. Since the TNF- α tip region is involved in the lectin-like activity of TNF- α , as demonstrated in the previous sections, the effect of the rabbit anti-mTNF- α tip polyclonal antibodies on LPS-induced septic shock *in vivo* was investigated. As shown in Table V, intraperitoneal injection of Balb/C mice (10 mice/group) with 100 μ g of protein G-purified anti-tip polyclonal antibodies, 2 h before the injection of the lethal dose of 500 μ g of LPS/mouse, resulted in an increased survival time as compared with the control mice that only received PBS or an irrelevant control polyclonal antibody preinjection (control IgG).

This result indicates that (pre)treatment of the animal with anti-m/hTNF- α tip antibodies can protect the animal from the lethal effects of the septic shock syndrome.

TABLE VI : Effect of lethal dose of LPS on survival time of mice

days post injection	mortality (%)		
	PBS	anti-tip	control IgG
0	0	0	0
1	40	0	40
2	100	20	100
3	100	50	100
4	100	80	100
5	100	80	100
6	100	100	100

EXAMPLE 5 : Tip-mutated or tip-deleted TNF- α is not hampered in its *in vitro* cytolytic/cytotoxic activity and has a reduced lethal effect

1. Recombinant and mutated mTNF- α and hTNF- α are cytotoxic to L-929 cells *in vitro*.

The purified recombinant mTNF- α and hTNF- α , produced in *E. coli*, had a specific activity of respectively, 2-3 10^8 Units/mg and 2-4 10^7 Units/mg as tested on specific cell lysis of L-929

cells in the 18 hours assay in the presence of Actinomycin D.

The mTNF- α tip-mutations or -deletion did not hamper the in vitro cytolytic activity of the molecule (Fig 11).

2. T(105), E(107), and/or E(110) mutated or tip deleted TNF- α has lost the mTNF- α trypanocidal activity.

The T(105), E(107), and/or E(110) mutants, combinations of these and deletions of the TPEGAE human or mouse TNF- α tip domain result in a significant loss of mTNF- α trypanocidal activity (Fig. 12). It is to be remarked in this context that, mutation of the P(106) and/or G(108) in the tip peptide to A did not change the trypanocidal nor the cytolytic activity of TNF- α (data not shown).

3. T(105), E(107) and/or E(110) tip-mutated or tip-deleted mTNF- α has a lower LD50 value then wild type mTNF- α .

Wild type mTNF- α is lethal for C57BL/6 mice at a dose of 10 μ g/mouse. It was tested if those mutations hampering the trypanocidal activity of mTNF- α (which could in previous examples be correlated with the lectin-like activity of the molecule) had any influence on the LD50 of the protein. As these TNF- α muteins of the present invention exert the same cell cytotoxic activity as wild type TNF- α and as this effect is directly linked with the in vivo tumoricidal activity of the cytokine, the TNF- α muteins of the invention could be useful as anti-tumor agents without the nefast toxic side effects of TNF- α . Moreover, any activity of TNF- α that could be linked with its lectin-like activity, such as induction of the septic shock syndrome, cachexia, the damage to endothelial cells, etc., could then be circumvented by using the above described TNF- α muteins.

Figure 13 shows the survival rate of intravenously injected C57BL/6 mice when treated with 10 μ g, 5 μ g, 2.5 μ g or 1 μ g/mouse of either wild type mTNF- α or T105A mTNF- α (6 mice/group). At the dose of 5 μ g/mouse a differential effect of the wild type and the T105A mTNF- α could be observed.

EXAMPLE 6 : The tip region of the TNF- α molecule has an effect on the in vivo half life of the molecule

Since lectin-carbohydrate interactions may modulate the clearance of a protein, the effect of mutations in the tip region on the in vivo half life of the molecule was tested. Female 8 weeks old C57bL/6 mice (6 mice/group - Charles Rivers Deutschland) were injected intraperitoneally with 1 μ g/mouse of either wild type mouse TNF- α or tip deleted TNF- α . Subsequently, the mice were bled 0.5, 5, 10, 15, 30, 60, 120, 180, or 240 minutes post-injection. The sera were tested for mTNF- α contents in the L-929 cytotoxic assay and in the indirect ELISA assay as detailed in the materials and methods sections detecting both wild type and tip mutated mouse TNF- α with the same sensitivity. The results of 2 experiments are given in Table VII below.

These experiments demonstrate a significant longer half life (at least 2 fold longer half life) for the tip deleted TNF- α mutant than for the wild type, TNF- α molecule.

Table VII: In vivo half life of mouse wild type and tip-deleted TNF- α .

Experiment 1

time post injection (minutes)	concentration TNF- α (ng/ml)	
	wild type TNF	tip deleted TNF
0.5	1000	1000
5	880	990
10	650	640
15	500	550
30	420	580
60	320	450
120	20	250
180	10	100
240	0	10

Experiment 2

time post injection (minutes)	concentration TNF- α (pg/ml)	
	wild type TNF	tip deleted TNF
0.5	1000	1000
15	550	600
30	500	500
60	480	500
120	20	330
180	0	240
240	0	20

EXAMPLE 7 : The tip region of the TNF- α molecule is involved in the induction of endothelial cells to produce inflammatory cytokines

1. Tip-mutated TNF- α induces less human interleukin-6 (IL6) on human microvascular endothelial cells of the brain than wild type TNF- α

Human microvascular endothelial cells of the brain (hMVECb) are prepared as described by Grau and Lou (1993).

The cells were plated in 24 well plates at 106 cells/ml and treated for 24 hours with various doses of wild type or tip-mutated TNF- α . Subsequently, the IL6 concentration in the supernatant of the cells was assessed using a commercially available hIL6 ELISA kit (Medgenix, Belgium). The results (shown in Table VIII) demonstrate that tip mutated TNF is less capable of inducing the endothelial brain cells to produce IL6.

Table VIII : IL6 production (pg/ml) by hMVECb cells by treatment with wild type mouse TNF- α or tip mutated mTNF- α

pg/ml		IL6 concentration (pg/ml)	
		experiment 1	experiment 2
wt mTNF:	0	620	800
	333	1520	1900
	1000	1250	1400
	1666	1220	1300
del mTNF:	0	620	800
	333	760	850
	1000	1180	1600
	1666	1240	1900
T105A mTNF:	0	620	800
	333	560	700
	1000	1080	1300
	1666	980	1250
P106A mTNF:	0	620	800
	333	720	1300
	1000	560	750
	1666	800	1300

2. The T105A and the P106A mTNF- α mutants induce less human interleukin 8 (IL8) than wild type TNF- α on human endothelial brain cells

The conditioned medium of 106 hMVECb cells/ml treated for 24 hours with different doses of wild type or tip mutated TNF- α was analysed on IL8 production using a commercially available human IL8 ELISA detection kit (Medgenix, Belgium). The results, shown in Table IX below, demonstrate that the T105A and P106A mutants are inducing about 3 fold less IL8 in these cells than wild type TNF- α while the tip deletion mutant was about two times more powerfull in inducing the inflammatory cytokine IL8.

Table IX : IL8 production (pg/ml) by hMVEC6 cells by treatment with wild type mouse TNF- α or tip-mutated mTNF- α

pg/ml		IL8 concentration (pg/ml)	
		experiment 1	experiment 2
wt mTNF:	0	40	80
	333	1050	1700
	1000	1680	1800
	1666	1200	1650
del mTNF:	0	40	80
	333	1800	2450
	1000	1300	2200
	1666	850	1250
T105A mTNF:	0	40	80
	333	600	750
	1000	800	1250
	1666	380	500
P106A mTNF:	0	40	80
	333	300	400
	1000	1100	1500
	1666	250	900

EXAMPLE 8 : The tip region of TNF- α is involved in the metastatis-promoting effect of the molecule

Besides the well defined anti-tumor effect of TNF- α , experiments concerning the influence of exogenous TNF- α on metastasis has been a point of discussion. In a murine fibrosarcoma model (Tomazic et al., 1988), a lung cancer model (Schlutz and Altom, 1990) and in melanoma models (Sylvester et al, 1990; Lollini et al., 1990) inhibition of formation of metastases by recombinant human TNF- α has been reported when the latter was given within 3 days of tumor cell inoculation. On the other hand, administration of recombinant human TNF- α led to an

increase of pulmonary metastasis when given 1 hour prior to the intravenous inoculation of a human melanoma cell line into nude mice (Giavazzi et al., 1990) or promoted the implantation of human ovarian cancer xenografts in the peritoneal cavity of nude mice (Malik et al., 1989).

The metastasis-promoting effect of wild type and tip deleted mouse TNF- α was compared by use of the 3LL-R tumorigenic Lewis lung carcinoma cell line (wt and del mTNF-resistant Lewis lung carcinoma) (Remels et al., 1989). Thereto, 8 weeks old female C57bl/6 mice (5 mice/group) (Charles River, Deutschland) were injected intraperitoneally with either PBS (control group) or 5 μ g of wild type or tip-deleted mouse TNF- α per mouse, 5 hours prior to the intravenous injection of $2 \cdot 10^6$ 3LL-R cells. This tumor inoculum normally leads to a 100% mortality 25 days after injection of the control group. After 17 days, the mice injected with wt mTNF/3LL-R all suffered from severe respiration problems whereas the mice in the del mTNF/3LL-R group looked significantly better and the mice in the PBS/3LL-R group looked completely healthy. At this time, all mice were killed and the lung weight as well as the amount of cancer cell nodules in the lung was assessed. The results are shown in Table X below. The weight of the lungs as well as the number of nodules present in the lungs was significantly higher (at least 100x) in the TNF- α treated mice indicating that TNF- α indeed is capable of enhancing the metastatic potential of TNF-resistant tumor cells. However, the metastatic enhancing potential of the TNF tip deletion mutant lays considerably lower than that of wild type TNF.

The observed metastasis-promoting effect for TNF- α might reside in an influence of TNF- α on the interaction of circulating tumor cells with the endothelium supporting the arrest, diapedesis and extravasation of tumor cells. As the tip-deleted TNF- α protein has significantly less metastasis promoting capacity than wild type TNF- α , the tip region of the molecule may interfere with the interaction of TNF- α with the endothelium. This interaction can be due to the enhanced expression of adhesion molecules on endothelial cells and/or tumor cells by

a direct action of $\text{TNF-}\alpha$ on these cells or being mediated via additional cells e.g. granulocytes or platelets.

Table X : Experimental metastasis inducing capacity of wild type and tip deleted $\text{TNF-}\alpha$ on TNF resistant 3LL-R lewis Lung carcinoma cells.

treatment	mean lung weight	# nodules in lung
control/3LL-R	153	0
PBS/3LL-R	141.6 ± 11.3	0.2 ± 0.4
wt mTNF/3LL-R	1088.2 ± 315.7	>50/>50/>50/>50/>50
del mTNF/3LL-R	566 ± 318	>50/>50/40/20/20

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SEQUENCE LISTING

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(ii) TITLE OF INVENTION: TNF-MUTEINS, A PROCESS FOR PREPARING THEM AND
THEIR USE AS ACTIVE SUBSTANCES IN PHARMACEUTICAL
COMPOSITIONS

(iii) NUMBER OF SEQUENCES: 34

(iv) COMPUTER READABLE FORM:

- (A) MEDIUM TYPE: Floppy disk
- (B) COMPUTER: IBM PC compatible
- (C) OPERATING SYSTEM: PC-DOS/MS-DOS
- (D) SOFTWARE: PatentIn Release #1.0, Version #1.25 (EPO)

(2) INFORMATION FOR SEQ ID NO: 1:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 14 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: YES

(iii) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 1:

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1				5						10			

(ix) FEATURE

(A) NAME/KEY: misc-feature

(B) LOCATION: 1

(D) OTHER INFORMATION: Xaa is Pro or Gln

(ix) FEATURE

(A) NAME/KEY: misc-feature

(B) LOCATION: 2

(D) OTHER INFORMATION: Xaa is Lys or Arg

(ix) FEATURE

(A) NAME/KEY: misc-feature

(B) LOCATION: 3

(D) OTHER INFORMATION: Xaa is Asp or Glu

(ix) FEATURE

(A) NAME/KEY: misc-feature

(B) LOCATION: 5

(D) OTHER INFORMATION: Xaa is Pro, Ala, Thr, or Gly

(ix) FEATURE

(A) NAME/KEY: misc-feature

(B) LOCATION: 7

(D) OTHER INFORMATION: Xaa is Gly, Ala, Thr, Pro, or Ser

(ix) FEATURE

(A) NAME/KEY: misc-feature

83

(B) LOCATION: 8

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(ix) FEATURE

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(B) LOCATION: 10

(D) OTHER INFORMATION: Xaa is Leu or Ala

(ix) FEATURE

(A) NAME/KEY: misc-feature

(B) LOCATION: 1..3

(D) OTHER INFORMATION: These amino acids may be present or absent

(ix) FEATURE

(A) NAME/KEY: misc-feature

(B) LOCATION: 10..14

(D) OTHER INFORMATION: These amino acids may be present or absent

(2) INFORMATION FOR SEQ ID NO: 2:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 4009 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: circular

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iii) ANTI-SENSE: NO

(vii) IMMEDIATE SOURCE:

(B) CLONE: pIG2

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 2:

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AAAAATTAAG CCCTGAAGAA GGGCAGGGGT ACCAGGAGGT TTAAATCATG GTAAGATCAA	240
GTAGTCAAAA TTCGAGTGAC AAGCCTGTAG CCCACGTCGT AGCAAACCAC CAAGTGGAGG	300
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AAGACAACCA ACTAGTGGTG CCAGCCGATG GGTGTGACCT TGTCTACTCC CAGGTTCTCT	420
TCAAGGGACA AGGCTGCCCC GACTACGTGC TCCTCACCCA CACCGTCAGC CGATTTGCTA	480
TCTCATACCA GGAGAAAGTC AACCTCCTCT CTGCCGTCAA GAGCCCCTGC CCCAAGGACA	540
CCCCTGAGGG GGCTGAGCTC AAACCCTGGT ATGAGCCCAT ATACCTGGGA GGAGTCTTCC	600
AGCTGGAGAA GGGGGACCAA CTCAGCGCTG AGGTCAATCT GCCCAAGTAC TTAGACTTTG	660
CGGAGTCCGG GCAGGTCTAC TTTGGAGTCA TTGCTCTGTG AAGGGAATGG GTGTTTCATCC	720
ATTCTCTACC CAGCCCCCAC TCTGACCCCT TTA CTCTGAC CCCTTTATTG TCTACTCCTC	780
AGAGCCCCCA GTCTGTGTCC TTCTAACTTA GAAAGGGGAT TATGGCTCAG AGTCCAACCTC	840
TGTGCTCAGA GCTTTCAACA ACTACTCAGA AACACAAGAT GCTGGGACAG TGACCTGGAC	900
TGTGGGCCTC TCATGCACCA CCATCAAGGA CTCAAATGGG CTTTCCGAAT TCTCTAGAGT	960
CGACCTGCAG CCCAAGCTTG GCTGTTTTTG CGGATGAGAG AAGATTTTCA GCCTGATACA	1020
GATTAAATCA GAACGCAGAA GCGGTCTGAT AAAACAGAAT TTGCCTGGCG GCAGTAGCGC	1080
GGTGGTCCCA CCTGACCCCA TGCCGAATC AGAAGTGAAA CGCCGTAGCG CCGATGGTAG	1140

TGTGGGGTCT	CCCCATGCGA	GAGTAGGGAA	CTGCCAGGCA	TCAAATAAAA	CGAAAGGCTC	1200
AGTCGAAAGA	CTGGGCCTTT	CGTTTTATCT	GTTGTTTGTC	GGTGAACGCT	CTCCTGAGTA	1260
GGACAAATCC	GCCGGGAGCG	GATTTGAACG	TTGCGAAGCA	ACGGCCCCGA	GGGTGGCGGG	1320
CAGGACGCCC	GCCATAAACT	GCCAGGCATC	AAATTAAGCA	GAAGGCCATC	CTGACGGATG	1380
GCCTTTTTGC	GTTTCTACAA	ACTCTTTTGT	TTATTTTTCT	AAATACATTC	AAATATGTAT	1440
CCGCTCATGA	GACAATAACC	CTGATAAATG	CTTCAATAAT	AAAAGGATCT	AGGTGAAGAT	1500
CCTTTTTGAT	AATCTCATGA	CCAAAATCCC	TTAACGTGAG	TTTTTCGTTCC	ACTGAGCGTC	1560
AGACCCCGTA	GAAAAGATCA	AAGGATCTTC	TTGAGATCCT	TTTTTTCTGC	GCGTAATCTG	1620
CTGCTTGCAA	ACAAAAAAC	CACCGCTACC	AGCGTGCGT	TGTTTGCCGG	ATCAAGAGCT	1680
ACCAACTCTT	TTTCCGAAGG	TAAGTGCTT	CAGCAGAGCG	CAGATACCAA	ATACTGTCCT	1740
TCTAGTGTAG	CCGTAGTTAG	GCCACCACTT	CAAGAACTCT	GTAGCACCGC	CTACATACCT	1800
CGCTCTGCTA	ATCCTGTTAC	CAGTGCTGTC	TGCCAGTGGC	GATAAGTCGT	GTCTTACCGG	1860
GTTGGACTCA	AGACGATAGT	TACCGGATAA	GGCGCAGCGG	TCGGGCTGAA	CGGGGGGTTT	1920
GTGCACACAG	CCCAGCTTGG	AGCGAACGAC	CTACACCGAA	CTGAGATACC	TACAGCGTGA	1980
GCATTGAGAA	AGCGCCACGC	TTCCCGAAGG	GAGAAAGGCG	GACAGGTATC	CGGTAAGCGG	2040
CAGGGTCGGA	ACAGGAGAGC	GCACGAGGGA	GCTTCCAGGG	GGAAACGCCT	GGTATCTTTA	2100
TAGTCCTGTC	GGGTTTCGCC	ACCTCTGACT	TGAGCGTCGA	TTTTTGTGAT	GCTCGTCAGG	2160
GGGGCGGAGC	CTATGGAAAA	ACGCCAGCAA	CGCGGCCTTT	TTACGGTTCC	TGGCCTTTTG	2220
CTGGCCTTTT	GCTCACATGT	TCTTCCTGTC	GTTATCCCCT	GATTCTGTGG	ATAACCGTAT	2280
TACCGCCTTT	GAGTGAGCTG	ATACCGCTCG	CCGCAGCCGA	ACGACCGAGC	GCAGCGAGTC	2340
AGTGAGCGAG	GAAGCGGAAG	AGCGCTGACT	TCCGCGTTTC	CAGACTTTAC	GAAACACGGA	2400
AACCGAAGAC	CATTCATGTT	GTTGCTCAGG	TCGCAGACGT	TTTGCAGCAG	CAGTCGCTTC	2460

ACGTTCCGCTC GCGTATCGGT GATTCAATTCT GCTAACCAGT AAGGCAACCC CGCCAGCCTA	2520
GCCGGGTCCT CAACGACAGG AGCAGCATCA TGCGCACCCG TGGCCAGGAC CCAACGCTGC	2580
CCGAGATGCG CCGCGTGCGG CTGCTGGAGA TGGCGGACGC GATGGATATG TTCTGCCAAG	2640
GGTTGGTTTG CGCATTACACA GTTCTCCGCA AGAATTGATT GGCTCCAATT CTTGGAGTGG	2700
TGAATCCGTT AGCGAGGTGC CGCCGGCTTC CATTCAGGTC GAGGTGGCCC GGCTCCATGC	2760
ACCGCGACGC AACGCGGGGA GGCAGACAAG GTATAGGGCG GCGCCTACAA TCCATGCCAA	2820
CCCGTTCCAT GTGCTCGCCG AGGCGGCATA AATCGCCGTG ACGATCAGCG GTCCAGTGAT	2880
CGAAGTTAGG CTGGTAAGAG CCGCGAGCGA TCCTTGAAGC TGTCCTGAT GGTCGTCATC	2940
TACCTGCCTG GACAGCATGG CCTGCAACGC GGCATCCCG ATGCCGCCGG AAGCGAGAAG	3000
AATCATAATG GGAAGGCCA TCCAGCCTCG CGTCGCGAAC GCCAGCAAGA CGTAGCCCAG	3060
CGCGTCGGCC GCCATGCCGG CGATAATGGC CTGCTTCTCG CCGAAACGTT TGGTGGCGGG	3120
ACCAAGTGACG AAGGCTTGAG CGAGGGCGTG CAAGATTCCG AATACCGCAA GCGACAGGCC	3180
GATCATCGTC GCGCTCCAGC GAAAGCGGTC CTCGCCGAAA ATGACCCAGA GCGCTGCCGG	3240
CACCTGTCTT ACGAGTTGCA TGATAAAGAA GACAGTCATA AGTGCGGCGA CGATAGTCAT	3300
GCCCCGCGCC CACCGGAAGG AGCTGACTGG GTTGAAGGCT CTCAAGGGCA TCGGTGACG	3360
CTCTCCCTTA TCGACTCCT GCATTAGGAA GCAGCCAGT AGTAGGTTGA GGCCGTTGAG	3420
CACCGCCGCC GCAAGGAATG GTGCATGCAA GGAGATGGCG CCCAACAGTC CCCCAGCCAC	3480
GGGGCCTGCC ACCATACCCA CGCCGAAACA AGCGCTCATG AGCCCGAAGT GGCGAGCCCG	3540
ATCTTCCCCA TCGGTGATGT CGGCGATATA GGCGCCAGCA ACCGCACCTG TGGCGCCGGT	3600
GATGCCGGCC ACGATGCGTC CGGCGTAGAG GATCCACAGG ACGGTGTGG TCGCCATGAT	3660
CGCGTAGTCG ATAGTGGCTC CAAGTAGCGA AGCGAGCAGG ACTGGGCGGC GGCCAAAGCG	3720
GTCGGACAGT GCTCCGAGAA CGGGTGCACA TAGAAATTGC ATCAACGCAT ATAGCGCTAG	3780

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CAGCACGCCA TAGTGACTGG CGATGCTGTC GGAATGGACG ATATCCCGCA AGAGGCCCGG 3840
CAGTACCGGC ATAACCAAGC CTATGCCTAC AGCATCCAGG GTGACGGTGC CGAGGATGAC 3900
GATGAGCGCA TTGTTAGATT TCATACACGG TGCCTGACTG CGTTAGCAAT TTAAGTGTGA 3960
TAAACTACCG CATTAAAGCT TATCGATGAT AAGCTGTCAA ACATGAGAA 4009

(2) INFORMATION FOR SEQ ID NO: 3:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 26 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iii) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 3:

CATGCTCAGA TCAAGTAGTC AAAATT 26

(2) INFORMATION FOR SEQ ID NO: 4:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 28 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iii) ANTI-SENSE: YES

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(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 4:

CGAATTTTGA CTACTTGATC TGAGCATG

28

(2) INFORMATION FOR SEQ ID NO: 5:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 27 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iii) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 5:

CAAGGACGCT CCGGAGGGGG CTGAGCT

27

(2) INFORMATION FOR SEQ ID NO: 6:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 19 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iii) ANTI-SENSE: YES

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 6:

CAGCCCCCTC CGGAGCGTC

19

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(2) INFORMATION FOR SEQ ID NO: 7:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 27 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iii) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 7:

CAAGGACACC GCTGAGGGCG CCGAGCT

27

(2) INFORMATION FOR SEQ ID NO: 8:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 19 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iii) ANTI-SENSE: YES

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 8:

CGGCGCCCTC AGCGGTGTC

19

(2) INFORMATION FOR SEQ ID NO: 9:

(i) SEQUENCE CHARACTERISTICS:

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- (A) LENGTH: 27 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iii) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 9:

CAAGGACACC CCTGCAGGCG CTGAGCT

27

(2) INFORMATION FOR SEQ ID NO: 10:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 19 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iii) ANTI-SENSE: YES

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 10:

CAGCGCCTGC AGGGGTGTC

19

(2) INFORMATION FOR SEQ ID NO: 11:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 27 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single

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(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iii) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 11:

CAAGGACACC CCTGAGGGCG CCGCCCT

27

(2) INFORMATION FOR SEQ ID NO: 12:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 23 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iii) ANTI-SENSE: YES

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 12:

AGGGCGGCGC CCTCAGGGGT GTC

23

(2) INFORMATION FOR SEQ ID NO: 13:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 27 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

92

(iii) HYPOTHETICAL: NO

(iii) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 13:

CAAGGACACC CCTGCAGGCG CCGCCCT

27

(2) INFORMATION FOR SEQ ID NO: 14:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 23 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iii) ANTI-SENSE: YES

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 14:

AGGGCGGCGC CTGCAGGGGT GTC

23

(2) INFORMATION FOR SEQ ID NO: 15:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 27 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iii) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 15:

CAAGGACGCT CCGGAGGGCG CCGCCCT

27

(2) INFORMATION FOR SEQ ID NO: 16:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 23 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iii) ANTI-SENSE: YES

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 16:

AGGGCGGGCGC CCTCCGAGC GTC

23

(2) INFORMATION FOR SEQ ID NO: 17:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 27 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iii) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 17:

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CAAGGATGCC CCGGCGGGTG CAGAGCT

27

(2) INFORMATION FOR SEQ ID NO: 18:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 19 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iii) ANTI-SENSE: YES

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 18:

CTGCACCCGC CGGGGCATC

19

(2) INFORMATION FOR SEQ ID NO: 19:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 27 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iii) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 19:

CAAGGATGCT CCGGCCGGTG CGGCCCT

27

(2) INFORMATION FOR SEQ ID NO: 20:

95

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 23 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iii) ANTI-SENSE: YES

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 20:

AGGGCCGCAC CGGCCGGAGC ATC

23

(2) INFORMATION FOR SEQ ID NO: 21:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 34 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iii) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 21:

CAAGGACCTC AAACCATGGT ATGAGCCCAT ATAC

34

(2) INFORMATION FOR SEQ ID NO: 22:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 26 base pairs
- (B) TYPE: nucleic acid

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- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

- (ii) MOLECULE TYPE: DNA (genomic)
- (iii) HYPOTHETICAL: NO
- (iii) ANTI-SENSE: YES

- (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 22:

ATGGGCTCAT ACCATGGTTT GAGGTC

26

- (2) INFORMATION FOR SEQ ID NO: 23:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 6 amino acids
 - (B) TYPE: amino acid
 - (D) TOPOLOGY: linear

- (ii) MOLECULE TYPE: peptide

- (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 23:

Thr	Pro	Glu	Gly	Ala	Glu
1				5	

- (2) INFORMATION FOR SEQ ID NO: 24:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 17 amino acids
 - (B) TYPE: amino acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

- (ii) MOLECULE TYPE: peptide

(ix) FEATURE:

- (A) NAME/KEY: Modified-site
- (B) LOCATION: 1
- (D) OTHER INFORMATION: /note= "Biotin-Gly-Gly is coupled to the N-terminus of the peptide"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 24:

Cys Gly Pro Lys Asp Thr Pro Glu Gly Ala Glu Leu Lys Pro Trp Tyr
1 5 10 15

Cys

(2) INFORMATION FOR SEQ ID NO: 25:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 17 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(ix) FEATURE:

- (A) NAME/KEY: Binding-site
- (B) LOCATION: 1
- (D) OTHER INFORMATION: /note= "Biotin-Gly-Gly is coupled to the N-terminus of the peptide"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 25:

Cys Gly Gln Arg Glu Thr Pro Glu Gly Ala Glu Ala Lys Pro Trp Tyr
1 5 10 15

Cys

(2) INFORMATION FOR SEQ ID NO: 26:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 8 amino acids
 - (B) TYPE: amino acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(ix) FEATURE:

- (A) NAME/KEY: Binding-site
- (B) LOCATION: 1
- (D) OTHER INFORMATION: /note= "Biotin-Gly-Gly is coupled to the N-terminus of the peptide"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 26:

Cys Thr Pro Glu Gly Ala Glu Cys

1

5

(2) INFORMATION FOR SEQ ID NO: 27:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 9 amino acids
 - (B) TYPE: amino acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(ix) FEATURE:

- (A) NAME/KEY: Binding-site
- (B) LOCATION: 1
- (D) OTHER INFORMATION: /note= "Biotin-Gly-Gly is coupled to the N-terminus of the peptide"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 27:

Glu Gly Ala Glu Leu Lys Pro Trp Tyr
1 5

(2) INFORMATION FOR SEQ ID NO: 28:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 3 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(ix) FEATURE:

- (A) NAME/KEY: Binding-site
- (B) LOCATION: 1
- (D) OTHER INFORMATION: /note= "Biotin-Gly-Gly is coupled to the N-terminus of the peptide"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 28:

Thr Pro Glu
1

(2) INFORMATION FOR SEQ ID NO: 29:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 8 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(ix) FEATURE:

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- (A) NAME/KEY: Modified-site
- (B) LOCATION: 1..8
- (D) OTHER INFORMATION: /note= "D amino acids"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 29:

Cys Thr Pro Glu Gly Ala Glu Cys
1 5

(2) INFORMATION FOR SEQ ID NO: 30:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 8 amino acids
 - (B) TYPE: amino acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(ix) FEATURE:

- (A) NAME/KEY: Binding-site
- (B) LOCATION: 1
- (D) OTHER INFORMATION: /note= "Biotin-Gly-Gly is coupled to the N-terminus of the peptide"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 30:

Cys Ala Pro Glu Gly Ala Glu Cys
1 5

(2) INFORMATION FOR SEQ ID NO: 31:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 8 amino acids
 - (B) TYPE: amino acid
 - (C) STRANDEDNESS: single

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(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(ix) FEATURE:

(A) NAME/KEY: Binding-site

(B) LOCATION: 1

(D) OTHER INFORMATION: /note= "Biotin-Gly-Gly is coupled
to the N-terminus of the peptide"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 31:

Cys Thr Ala Glu Gly Ala Glu Cys
1 5

(2) INFORMATION FOR SEQ ID NO: 32:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 8 amino acids

(B) TYPE: amino acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(ix) FEATURE:

(A) NAME/KEY: Binding-site

(B) LOCATION: 1

(D) OTHER INFORMATION: /note= "Biotin-Gly-Gly is coupled
to the N-terminus of the peptide"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 32:

Cys Thr Pro Ala Gly Ala Glu Cys
1 5

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(2) INFORMATION FOR SEQ ID NO: 33:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 8 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(ix) FEATURE:

- (A) NAME/KEY: Binding-site
- (B) LOCATION: 1
- (D) OTHER INFORMATION: /note= "Biotin-Gly-Gly is coupled to the N-terminus of the peptide"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 33:

Cys Thr Pro Glu Ala Ala Glu Cys

1

5

(2) INFORMATION FOR SEQ ID NO: 34:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 8 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(ix) FEATURE:

- (A) NAME/KEY: Binding-site
- (B) LOCATION: 1
- (D) OTHER INFORMATION: /note= "Biotin-Gly-Gly is coupled to the N-terminus of the peptide"

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(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 34:

Cys	Thr	Pro	Glu	Gly	Ala	Ala	Cys
1				5			

CLAIMS

1. A tumor necrosis factor mutein characterized in that the TNF- α amino acid sequence is mutated, or deleted totally or partially, in the region extending from amino acid position at 101 to 116 in such a way that:

- either the lectin-like activities are modulated with respect to TNF- α ,
- and/or the toxic activity is reduced with respect to TNF- α ,
- and/or the inflammatory cytokines inducing capacities are modulated with respect to TNF- α ,
- and/or the adhesion molecules inducing capacities are modulated with respect to TNF- α ,
- and/or the metastasis promoting activity is reduced with respect to TNF- α ,

and providing that these TNF- α muteins have preferentially retained the tumoricidal activity of TNF- α , and providing that said TNF muteins are different from human TNF- α wherein amino acids 1 to 8 are replaced by a sequence within the region spanning amino acids 5 to 30 of laminin, and providing that said TNF muteins are different from human TNF- α wherein:

amino acid position 101 is Ser, hTNF- α wherein amino acid position 102 is Arg or deleted, hTNF- α wherein amino acid position 103 is Trp, hTNF- α wherein amino acid position 105 is Pro, hTNF- α wherein amino acid position 105 is Ile, hTNF- α wherein amino acid position 105 is Ile and position 44 is Cys, hTNF- α wherein amino acid position 106 is Ser, hTNF- α wherein amino acid position 106 is Ser and position 131 is Cys, hTNF- α wherein amino acid position 108 is Phe, hTNF- α wherein amino acid position 110 is Lys, hTNF- α wherein amino acid positions 111 to 112 are deleted, hTNF- α wherein amino acid position 112 is deleted or Met, hTNF- α wherein amino acid position 111 is deleted and amino acid positions 109 and 120 are respectively Gln and His, hTNF- α wherein amino acid position 115 is Ile or Cys, hTNF- α

wherein amino acid position 116 is Lys, His or Val, hTNF- α wherein amino acid positions 115-116 are Ile-Lys; and with said TNF muteins possibly containing in their peptidic chain outside the region spanning amino acids 101 to 116 of TNF- α , additional modifications consisting of substitutions and/or deletions and/or additions of one or several amino acid residues, and with said muteins being characterized in that they have retained the aforementioned activities; or a pharmaceutically acceptable salt thereof.

2. A TNF mutein according to claim 1, further characterized in that the lectin-like activities are modulated with respect to TNF- α .

3. A TNF mutein according to any of claims 1 or 2, further characterized in that the lectin-like activities are increased with respect to TNF- α .

4. A TNF mutein according to any of claims 1 or 2, further characterized in that the lectin-like activities are reduced with respect to TNF- α .

5. A TNF mutein according to claim 1, further characterized in that the toxic activity is reduced with respect to TNF- α .

6. A TNF mutein according to claim 1, further characterized in that the inflammatory cytokine inducing capacities are modulated with respect to TNF- α .

7. A TNF mutein according to any of claims 1 or 6, further characterized in that the inflammatory cytokine inducing capacities are increased with respect to TNF- α .

8. A TNF mutein according to any of claims 1 or 6, further characterized in that the inflammatory cytokine inducing capacities are reduced with respect to TNF- α .

9. A TNF mutein according to claim 1, further characterized in that the adhesion molecule inducing capacities are modulated with respect to TNF- α .

10. A TNF mutein according to any of claims 1 or 9, further characterized in that the adhesion molecule inducing capacities are reduced with respect to TNF- α .

11. A TNF mutein according to any of claims 1 or 9, further characterized in that the adhesion molecule inducing capacities are increased with respect to TNF- α .

12. A TNF mutein according to claim 1, further characterized in that the metastasis promoting activity is reduced with respect to TNF- α .

13. A TNF mutein according to any of claims 1 to 12, further characterized in that the tumoricidal activity is retained with respect to TNF- α .

14. A TNF mutein according to any of claims 1 to 12, further characterized in that the tumoricidal activity is reduced with respect to TNF- α .

15. A TNF mutein according to any of claims 1 to 14, further characterized in it shows an increased half life time with respect to TNF- α .

16. A TNF mutein according to any of claims 1 to 15, characterized in that at least part of the region extending from amino acid positions 101 to 116 of TNF- α , or the complete region corresponding to amino acid positions 101 to 116 of TNF- α has been deleted, and preferably at least the region covering amino acid positions 105 to 110 has been deleted.

17. A TNF mutein according to any of claims 1 to 15, characterized in that at least one of the amino acids in the region extending from amino acids 101 to 116 of TNF- α , and preferably at least one of the amino acids in the region extending from amino acids 105 to 110, has been mutated or deleted.

18. A Nucleic acid sequence encoding any of the polypeptides according to claims 1 to 17.

19. A process for the preparation of the polypeptides according to any of claims 1 to 17, comprising the steps of:

- transformation of an appropriate cellular host with a vector, particularly a plasmid, a cosmid, a phage or a virus, in which a nucleic acid sequence according to claim 18 coding for at least one of the polypeptides according to any of claims 1 to 17 has been inserted (insert) under the control of the appropriate regulatory elements, particularly a promoter recognized by the polymerases of the cellular host and, in the case of a procaryotic host, an appropriate ribosome binding site (RBS), enabling the expression in said cellular host of said nucleic acid sequence,

- culture of said transformed cellular host under conditions enabling the expression of said insert.

20. A TNF mutein according to any of claims 1 to 17, for treating illnesses and pathological conditions such as, sepsis, septic shock, Gram negative sepsis, endo-toxic shock, toxic shock syndrome, cachexia, microbial infections, rheumatoid arthritis, inflammatory conditions, respiratory distress syndrome, pulmonary fibrosis, infections, graft-versus-host-disease, reperfusion damage such as myocardial ischaemia, AIDS, cancer, cerebral malaria, immunosuppression, etc.

21. A pharmaceutical composition, containing as active substance, at least anyone of the TNF mutein polypeptides according to any

of claims 1 to 17, in association with a pharmaceutical acceptable vehicle.

22. Use of a TNF mutein characterized in that the TNF- α amino acid sequence is mutated, or deleted totally or partially, in the region extending from amino acid position at 101 to 116 in such a way that:

- either the lectin-like activities are modulated with respect to TNF- α ,
- and/or the toxic activity is reduced with respect to TNF- α ,
- and/or the inflammatory cytokines inducing capacities are modulated with respect to TNF- α ,
- and/or the adhesion molecules inducing capacities are modulated with respect to TNF- α ,
- and/or the metastasis promoting activity is reduced with respect to TNF- α ,

- and/or show an increased half life time with respect to TNF- α , and providing that these TNF- α muteins have preferentially retained the tumoricidal activity of TNF- α , and with said TNF muteins possibly containing in their peptidic chain outside amino acid region 101 to 116 of TNF- α , additional modifications consisting of substitutions and/or deletions and/or additions of one or several amino acid residues, and with said muteins being characterized in that they have retained the aforementioned activities; or a pharmaceutically acceptable salt thereof, for the preparation of a medicament for treating illnesses and pathological conditions, such as, sepsis, septic shock, Gram negative sepsis, endo-toxic shock, toxic shock syndrome, cachexia, microbial infections, rheumatoid arthritis, inflammatory conditions, respiratory distress syndrome, pulmonary fibrosis, infections, graft-versus-host-disease, reperfusion damage such as myocardial ischaemia, AIDS, cancer, cerebral malaria, immunosuppression, etc.

23. Use of an antibody specifically detecting an epitope residing in the region comprising amino acids 101 to 116 of TNF- α , more

particularly a monoclonal antibody, characterized in that it:

- either modulates the lectin-like activities of TNF- α ,
 - and/or inhibits the toxic activity of TNF- α ,
 - and/or modulates the inflammatory cytokines inducing capacities of TNF- α ,
 - and/or modulates the adhesion molecules inducing capacities of TNF- α ,
 - and/or inhibits the metastasis promoting activity of TNF- α ,
- for the preparation of a medicament for treating TNF-induced septic shock or illnesses or pathological conditions associated with the in vivo activities of TNF- α .

24. Use of an immunological complex comprising a monoclonal antibody according to claim 23, and complete TNF- α , for the preparation of a medicament for treating illnesses such as tumors.

25. Use of an antisense peptide of a peptide comprising at least part of the region 101 to 116 of TNF- α , more particularly a monoclonal antibody, characterized in that it :

- either modulates the lectin-like activities of TNF- α ,
 - and/or inhibits the toxic activity of TNF- α ,
 - and/or modulates the inflammatory cytokines inducing capacities of TNF- α ,
 - and/or modulates the adhesion molecules inducing capacities of TNF- α ,
 - and/or inhibits the metastasis promoting activity of TNF- α ,
- for the preparation of a medicament for treating TNF-induced septic shock or illnesses or pathological conditions associated with the lectin-like effects of TNF- α .

26. Use of a complex comprising an antisense peptides according to claim 25 and TNF- α for the preparation of a medicament for treating illnesses such as tumors.

27. Use of an antibody or antisense peptide according to any of

claims 23 to 26, characterized in that said antibody or said peptide modulates the lectin-like activities of TNF- α .

28. Use of an antibody or antisense peptide according to any of claims 23 to 26, characterized in that said antibody or said peptide inhibits the lectin-like activities of TNF- α .

29. Use of an antibody or antisense peptide according to any of claims 23 to 26, characterized in that said antibody or said peptide stimulates the lectin-like activities of TNF- α .

30. Use of an antibody or antisense peptide according to any of claims 23 to 26, characterized in that said antibody or said peptide inhibits the toxic activity of TNF- α .

31. Use of an antibody or antisense peptide according to any of claims 23 to 26, characterized in that said antibody or said peptide modulates the inflammatory cytokines inducing capacities of TNF- α .

32. Use of an antibody or antisense peptide according to any of claims 23 to 26, characterized in that said antibody or said peptide inhibits the inflammatory cytokines inducing capacities of TNF- α .

33. Use of an antibody or antisense peptide according to any of claims 23 to 26, characterized in that said antibody or said peptide modulates the adhesion molecules inducing capacities of TNF- α .

34. Use of an antibody or antisense peptide according to any of claims 23 to 26, characterized in that said antibody or said peptide inhibits the adhesion molecules inducing capacities of TNF- α .

35. Use of an antibody or antisense peptide according to any of

claims 23 to 26, characterized in that said antibody or said peptide stimulates the adhesion molecules inducing capacities of TNF- α .

36. Use of an antibody or antisense peptide according to any of claims 23 to 26, characterized in that said antibody or said peptide inhibits the metastasis promoting activity TNF- α .

37. Use of an antibody or antisense peptide according to any of claims 24 or 26, characterized in that said antibody or said peptide increases the half life time of TNF- α .

38. Cells transfected with a nucleic acid according to claim 18 coding for the TNF muteins according to any of claims 1 to 17, said nucleic acid being inserted into any suitable vector, with said cells being preferably autologous cells derived from the patient (e.g. a cancer patient) to be treated with such compositions; and with said vector-insert combination being constructed in such a way as to allow continuous expression of the TNF mutein at either a constant level, or at a level which can be modified, depending on the exact nature of the vector used to make the vector-insert combination.

39. Pharmaceutical composition containing as active substance transfected cells according to claim 38, in association with a pharmaceutical acceptable vehicle.

40. Use of transfected cells according to claim 38, for the preparation of a medicament for treating illnesses such as cancer.

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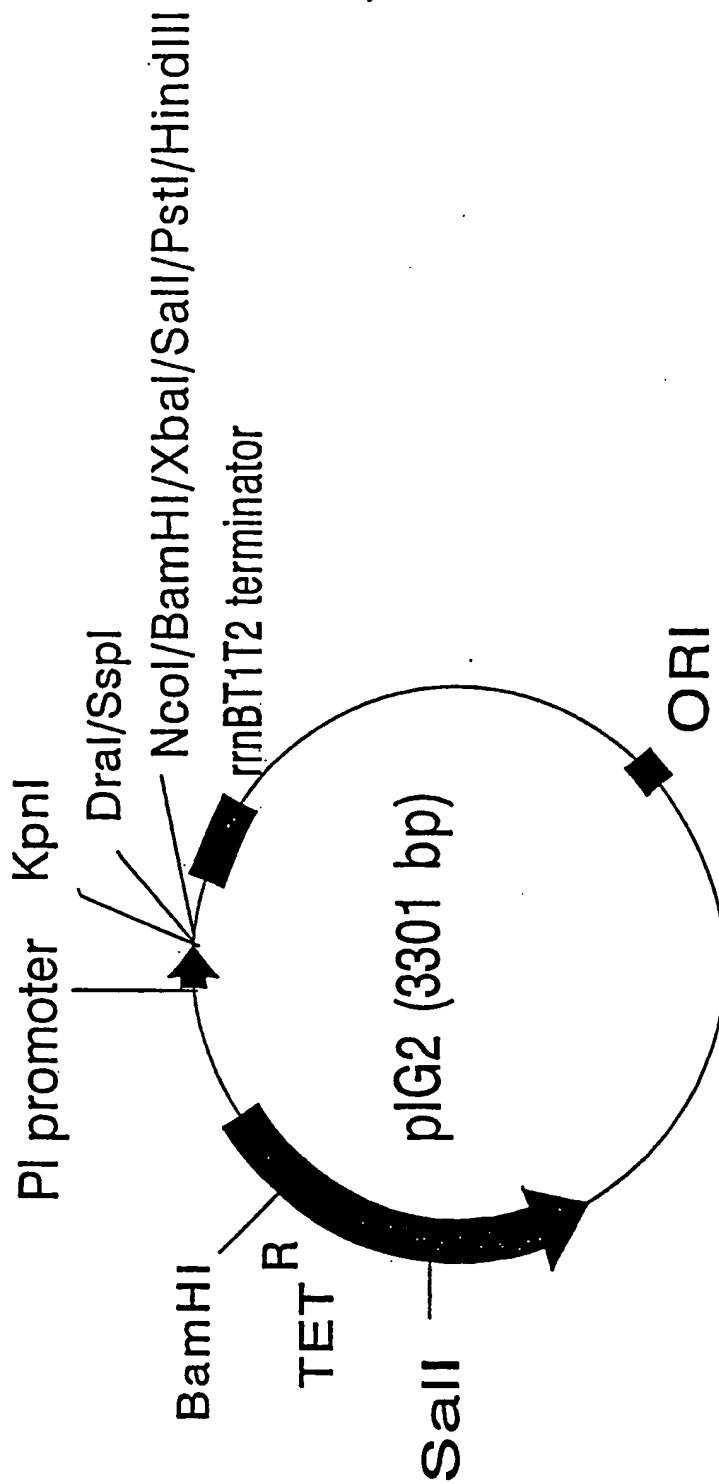


Figure 1A

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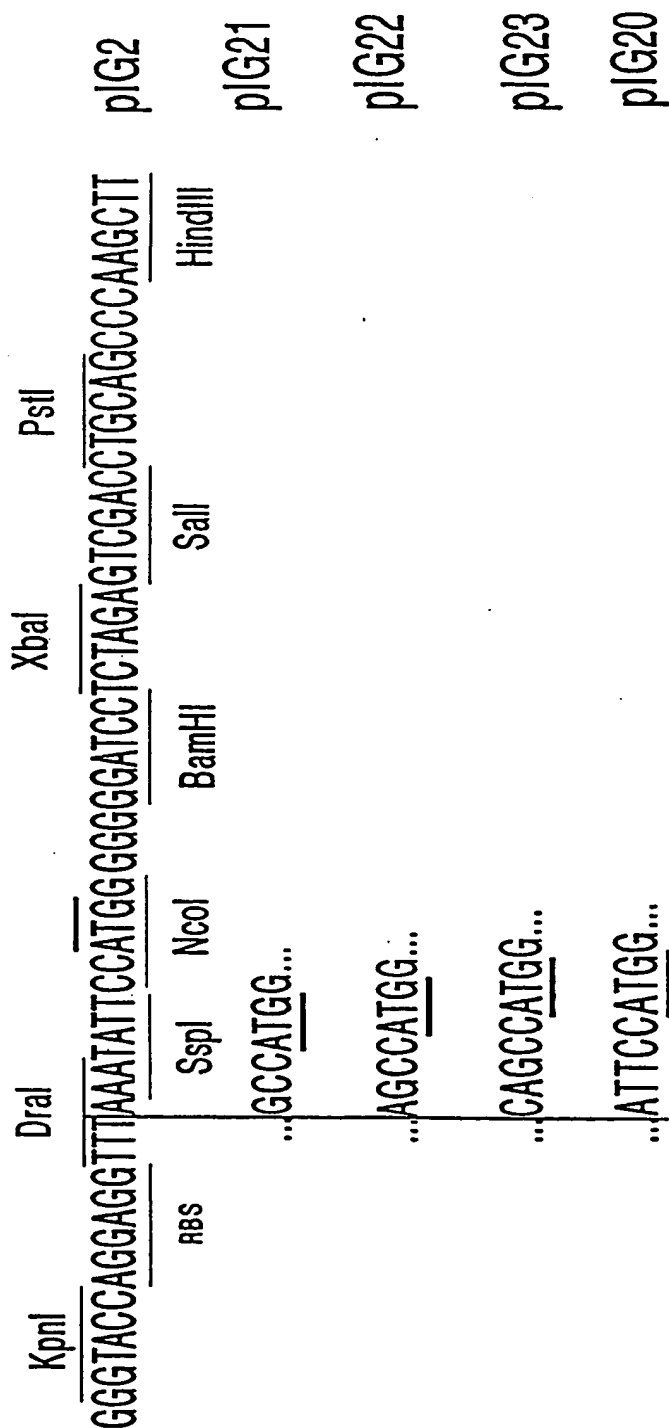


Figure 1B

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TTCCGGGGAT CTCTCACCTA CCAACAATG CCCCCCTGCA AAAAATAAAT TCATATAAAA 60
AACATAACAG TAACCATCTG CGGTGATAAA TTATCTCTGG CCGTGTGAC ATAAATACCA 120
CTGGCGGTGA TACTGAGCAC ATCAGCAGGA CGCACTGACC ACCATGAAGG TGACGCTCTT 180
AAAAATTAAAG CCTGAAGAA GGGCAGGGGT ACCAGGAGGT TTAAATATTC CATGGGGGGG 240
ATCCTCTAGA GTCGACCTGC AGCCCAAGCT TGGCTGTTTT GGCGGATGAG AGAAGATTTT 300
CAGCCTGATA CAGATTAAAT CAGAACGCAG AAGCGGTCTG ATAAACAGA ATTTGCCCTGG 360
CGGCAGTAGC GCGGTGGTCC CACCTGACCC CATGCCGAAC TCAGAAAGTGA AACGCCGTAG 420
CGCCGATGGT AGTGTGGGT CTCCCCATGC GAGAGTAGGG AACTGCCAGG CATCAAATAA 480
AACGAAAGGC TCAGTCGAAA GACTGGGCCT TTCGTTTTAT CTGTTGTTG TCGGTGAACG 540
CTCTCCTGAG TAGGACAAAT CCGCCGGGAG CGGATTGAA CGTTGCCGAG CAACGGCCCG 600
GAGGGTGGCG GGCAGGACGC CCGCCATAAA CTGCCAGGCA TCAAAATTAAG CAGAAGGCCA 660
TCCTGACGGA TGGCCTTTTT GCGTTTCTAC AAACCTTTTT GTTTATTTTT CTAAATACAT 720
TCAAATATGT ATCCGCTCAT GAGACAATAA CCCTGATAAA TGCTTCAATA ATAAAGGAT 780
CTAGGTGAAG ATCCTTTTIG ATAATCTCAT GACCAAATC CCTTAACGTG AGTTTTCGTT 840
CCACTGAGCG TCAGACCCCG TAGAAAAGAT CAAAGGATCT TCTTGAGATC CTTTTTTTCT 900
GCGCGTAATC TGCTGCTTGC AAACAAAAAA ACCACCGCTA CCAGCGGTGG TTTGTTTGCC 960

Figure 2

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GGATCAAGAG CTACCAACTC TTTTTCGGAA GGTAAGTGGC TTCAGCAGAG CGCAGATACC	1020
AAATACTGTC CTTCTAGTGT AGCCGTAGTT AGGCCACCAC TTCAAGAACT CTGTAGCACC	1080
GCCTACATAC CTCGCTCTGC TAATCCTGTT ACCAGTGGCT GCTGCCAGTG GCGATAAGTC	1140
GTGTCTTACC GGGTTGGA CTCAAGACGATA GTTACCGGAT AAGCGCAGC GGTCTGGGCTG	1200
AACGGGGGGT TCGTGCACAC AGCCCAGCTT GGAGCGAAGC ACCTACACCG AACTGAGATA	1260
CCTACAGCGT GAGCATTGAG AAAGCGCCAC GCTTCCCGAA GGGAGAAAGG CGGACAGGTA	1320
TCCGGTAAGC GGCAGGGTCG GAACAGGAGA GCGCACGAGG GAGCTTCCAG GGGGAAACGC	1380
CTGGTATCTT TATAGTCCGTG TCGGGTTTCG CCACCTCTGA CTTGAGCGTC GATTTTGTG	1440
ATGCTCGTCA GGGGGCGGA GCCTATGGAA AAACGCCAGC AACCGGCCCT TTTTACGGTT	1500
CCTGGCCCTT TGCTGGCCTT TTGCTCACAT GTTCTTTCTT GCGTTATCCC CTGATTCTGT	1560
GGATAACCGT ATTACCGCCT TTGAGTGAGC TGATACCGCT CGCCGCAGCC GAACGACCGA	1620
GCGCAGCGAG TCAGTGAGCG AGGAAGCGGA AGAGCGCTGA CTTCCGCGTT TCCAGACTTT	1680
ACGAAACACG GAAACCGAAG ACCATTATG TTGTTGCTCA GGTCCGAGAC GTTTTGCAGC	1740
AGCAGTCGCT TCACGTTTCG TCGCGTATCG GTGATTTCATT CTGCTAACCA GTAAGGCAAC	1800
CCCGCCAGCC TAGCCGGGTC CTCAACGACA GGAGCAGCAT CATCGGCACC CGTGGCCAGG	1860
ACCCAACGCT GCCCGAGATG CGCCGCGTGC GGCTGCTGGA GATGGCGGAC GCGATGGATA	1920

Figure 2 - Continuation 1

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TGTTCTGCCA	AGGGTTGGTT	TGCGCATTCA	CAGTTCTCCG	CAAGAAATTGA	TTGGCTCCAA	1980
TTCTTTGGAGT	GGTGAATCCG	TTAGCGAGGT	GCCGCCGGCT	TCCATTCAGG	TCGAGGTGGC	2040
CCGGCTCCAT	GCACCGCGAC	GCAACGGGG	GAGGCAGACA	AGGTATAGGG	CGGCGCCTAC	2100
AATCCATGCC	AACCCGTTCC	ATGTGCTCGC	CGAGGCGGCA	TAAATCGCCG	TGACGATCAG	2160
CGGTCCAGTG	ATCGAAGTTA	GGCTGGTAAG	AGCCGCGAGC	GATCCTTGAA	GCTGTCCCTG	2220
ATGGTCGTCA	TCTACCTGCC	TGGACAGCAT	GGCCTGCAAC	GCGGGCATCC	CGATGCCGCC	2280
GGAAGCGAGA	AGAAATCATAA	TGGGGAAGGC	CATCCAGCCT	CGCGTCGCGA	ACGCCAGCAA	2340
GACGTAGCCC	AGCGCGTCGG	CCGCCATGCC	GGCGATAATG	GCCTGCTTCT	CGCCGAAAACG	2400
TTTGTTGGCG	GGACCAGTGA	CGAAGGCTTG	AGCGAGGGCG	TGCAAGATTC	CGAATACCGC	2460
AAGCGACAGG	CCGATCATCG	TCGCGCTCCA	GCGAAAGCGG	TCCTCGCCGA	AAATGACCCA	2520
GAGCGCTGCC	GGCACCTGTC	CTACGAGTTG	CATGATAAAG	AAGACAGTCA	TAAGTGCGGC	2580
GACGATAGTC	ATGCCCCCGG	CCCACCGGAA	GGAGCTGACT	GGGTTGAAGG	CTCTCAAGGG	2640
CATCGGTCGA	CGCTCTCCCT	TATGCGACTC	CTGCATTAGG	AAGCAGCCCA	GTAGTAGGTT	2700
GAGGCCGTTG	AGCACCGCCG	CCGCAAGGAA	TGGTGCATGC	AAGGAGATGG	CGCCCCAACAG	2760
TCCCCCGGCC	ACGGGGCCTG	CCACCATACC	CACGCCGAAA	CAAGCGCTCA	TGAGCCCGAA	2820
GTGGCGAGCC	CGATCTTCCC	CATCGGTGAT	GTCGGCGATA	TAGGCGCCAG	CAACCGCACC	2880

Figure 2 - Continuation 2

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TGTGGCGCG GTGATGCCGG CCACGATGCG TCCGGCGTAG AGGATCCACA GGACGGGTGT 2940
 GGTCGCCATG ATCGCGTAGT CGATAGTGGC TCCAAGTAGC GAAGCGAGCA GGA CTGGGCG 3000
 GCGGCCAAAG CCGTCGGACA GTGCTCCGAG AACGGGTGCG CATAGAAATT GCATCAACGC 3060
 ATATAGCGCT AGCAGCACGC CATAGTGACT GCGGATGCTG TCGGAATGGA CGATATCCCG 3120
 CAAGAGGCCC GGCAGTACCG GCATAACCAA GCCTATGCCT ACAGCATCCA GGGTGACGGT 3180
 GCCGAGGATG ACGATGAGCG CATTGTTAGA TTTCATACAC GGTGCCTGAC TCGGTTAGCA 3240
 ATTTAACTGT GATAAACTAC CGCATFAAG CTTATCGATG ATAAGCTGTC AAACATGAGA 3300
 GCGCGCGCC CACCGGAGG AGCTGACTGG GTTGAAGGCT CTCAGGGCA TCGTCTGACG 3360
 CTCTCCCTTA TCGACTCCT GCATTAGGAA GCAGCCCAGT AGTAGGTGA GGCGGTGAG 3420
 CACCGCGCC GCAAGGAATG GTGCTATGCA GGAGATGGCG CCAACAGTC CCCCGGCCAC 3480
 GGGCCTGCC ACCATACCCA CGCGGAAACA AGCGTCTATG AGCCCGAAGT GCGAGCCCCG 3540
 ATCTTCCCCA TCGGTGATGT CGGCGATATA GGCGCCAGCA ACCGCACCTG TGGCGCCGGT 3600
 GATCCCGCC ACGATCGTC CGGCGTAGAG GATCCACAGG ACGGGTGTGG TCGCCATGAT 3660
 CGCGTAGTCG ATAGTGGCTC CAAGTAGCGA AGCGAGCAGG ACTGGGCGGC GGCCAAAGCG 3720
 GTCGACAGT GCTCCGAGAA CCGGTGCGCA TAGAATTGC ATCAACGCAT ATAGCGCTAG 3780
 CAGCAGCCA TAGTACTGG CGATGCTGTC GGAATGGAGC ATATCCCGCA AGAGGCCCGG 3840
 CAGTACCGC ATACCCAGC CTATGCCTAC AGCATCCAGG GTGACGGTGC CGAGGATGAC 3900
 GATGAGCGCA TTGTTAGATT TCATACACGG TGCCTGACTG CGTTAGCAAT TTAACGTGA 3960
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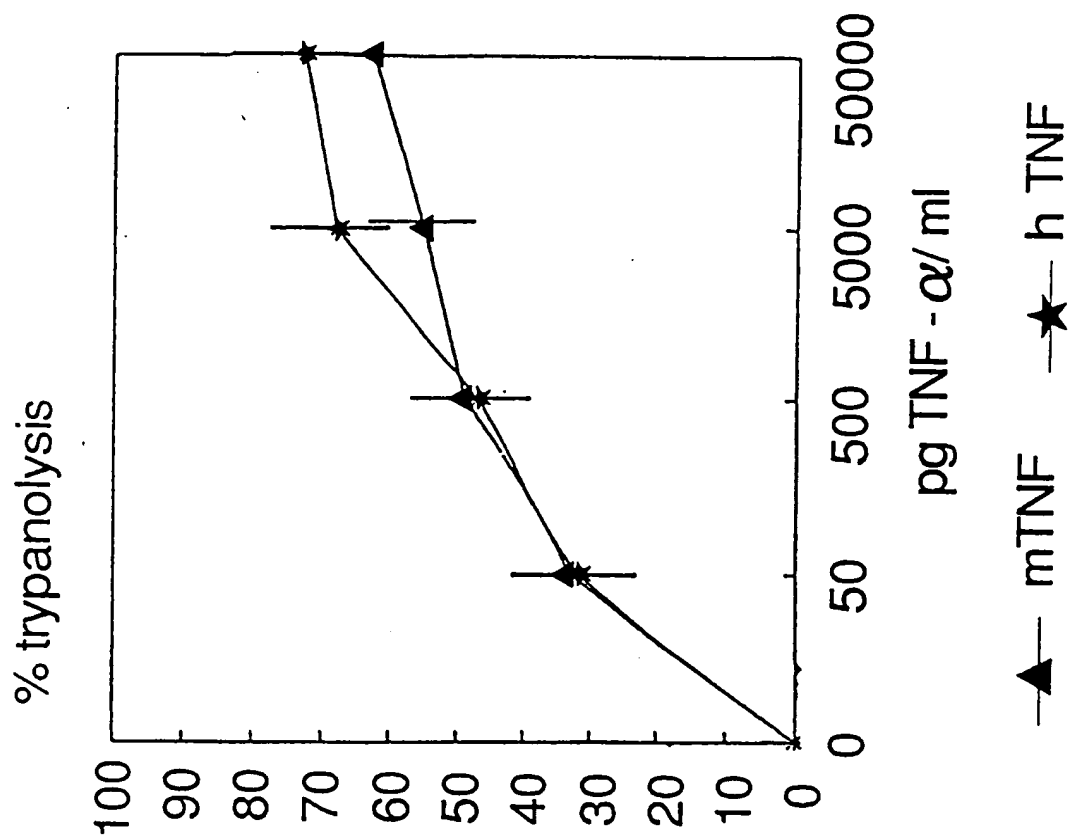


Figure 3A

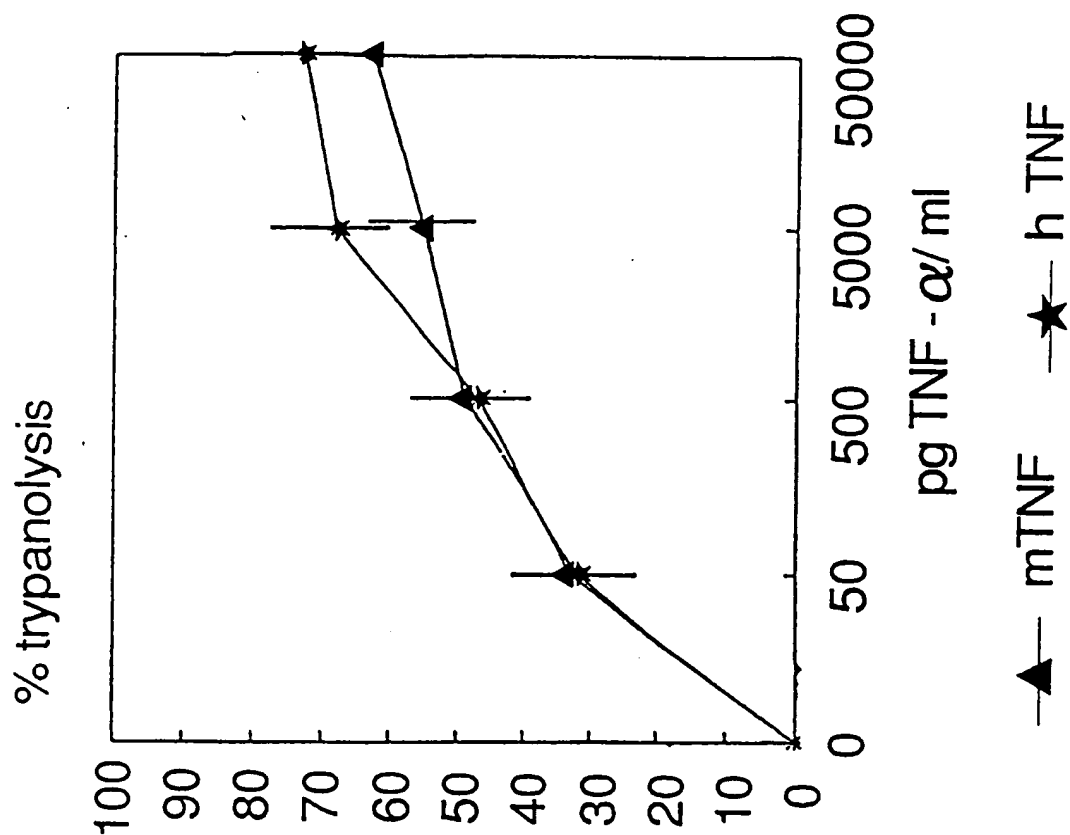


Figure 3B

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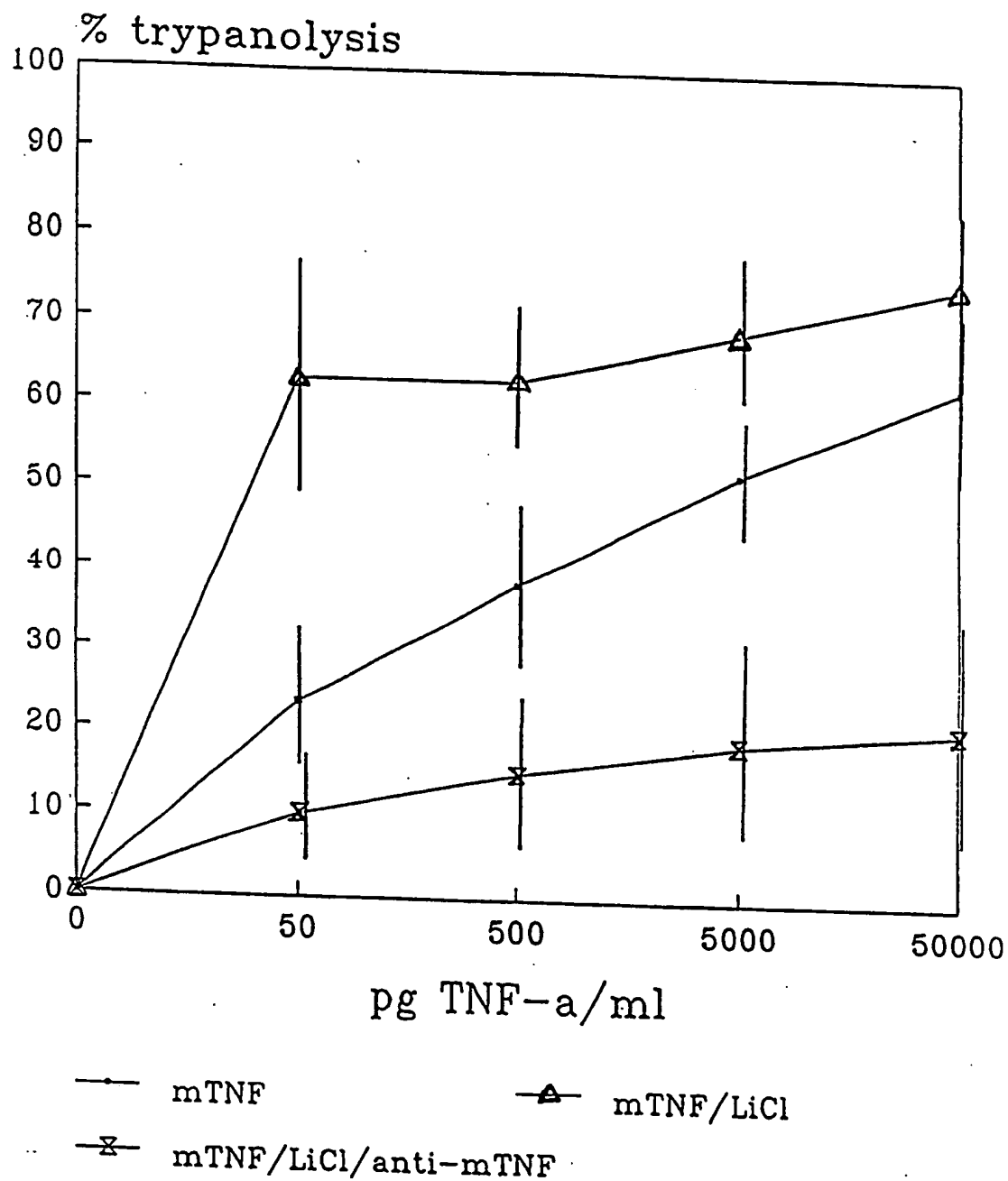


Figure 4

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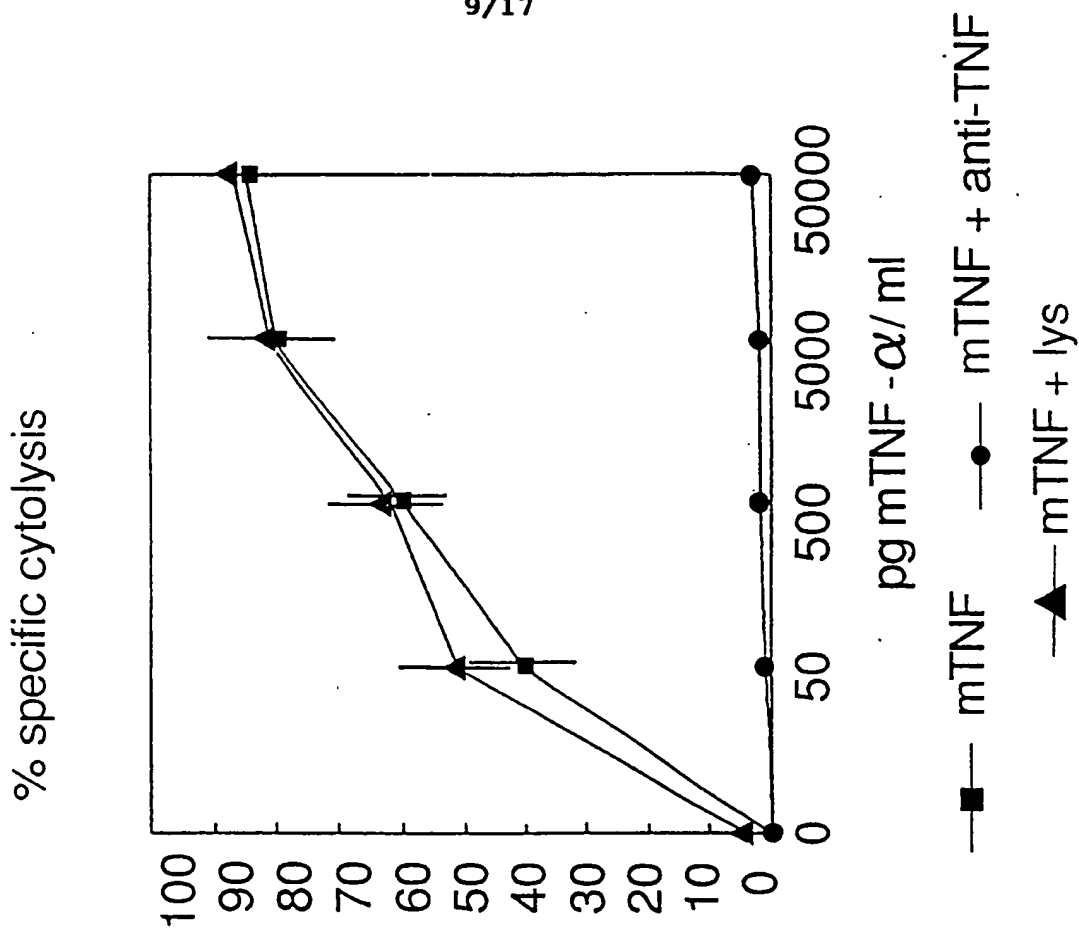


Figure 5B

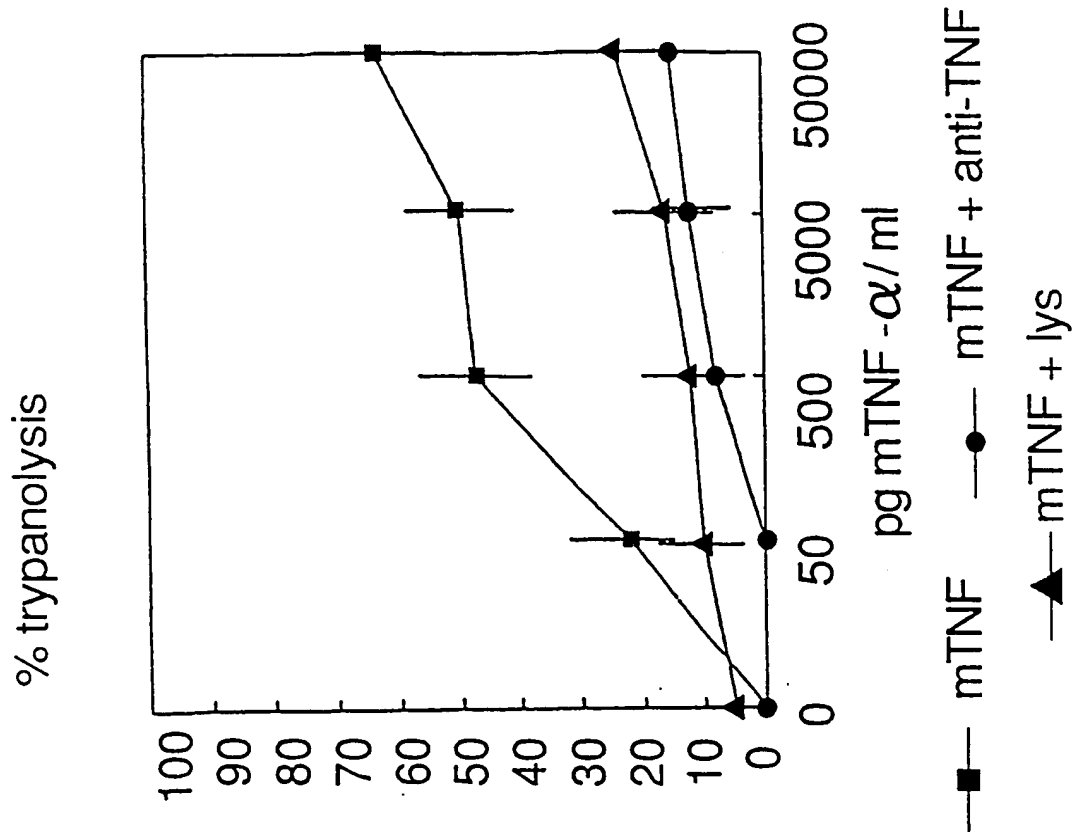


Figure 5A

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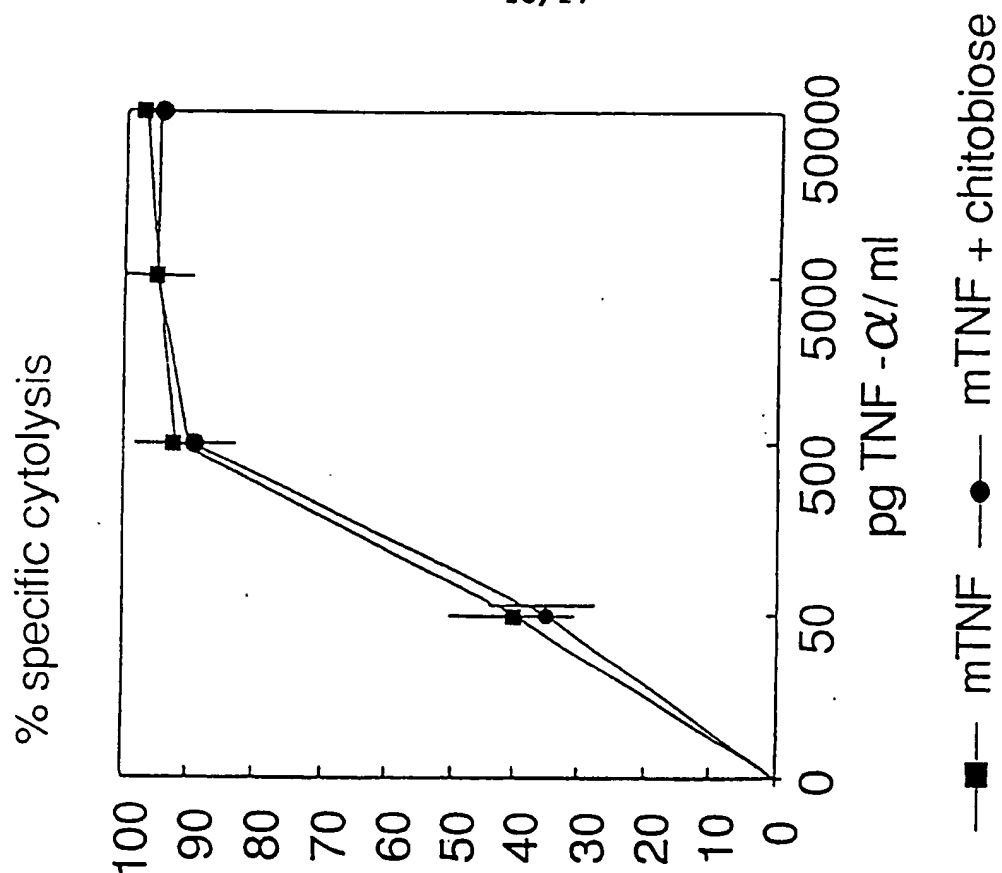


Figure 6A

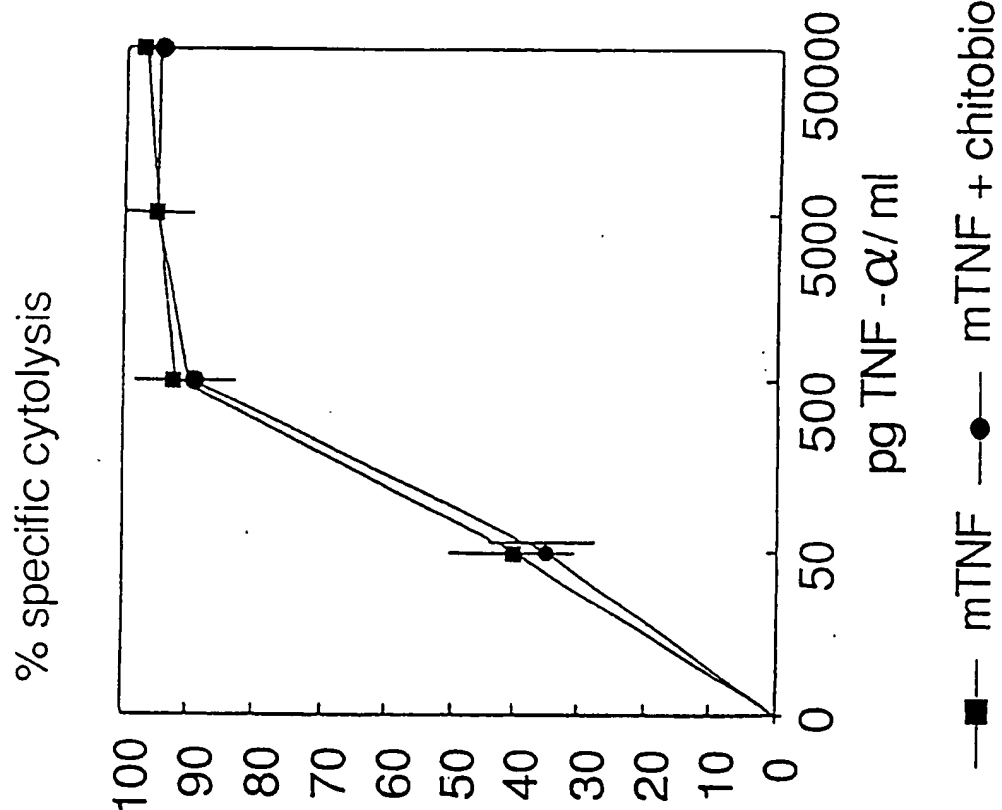


Figure 6B

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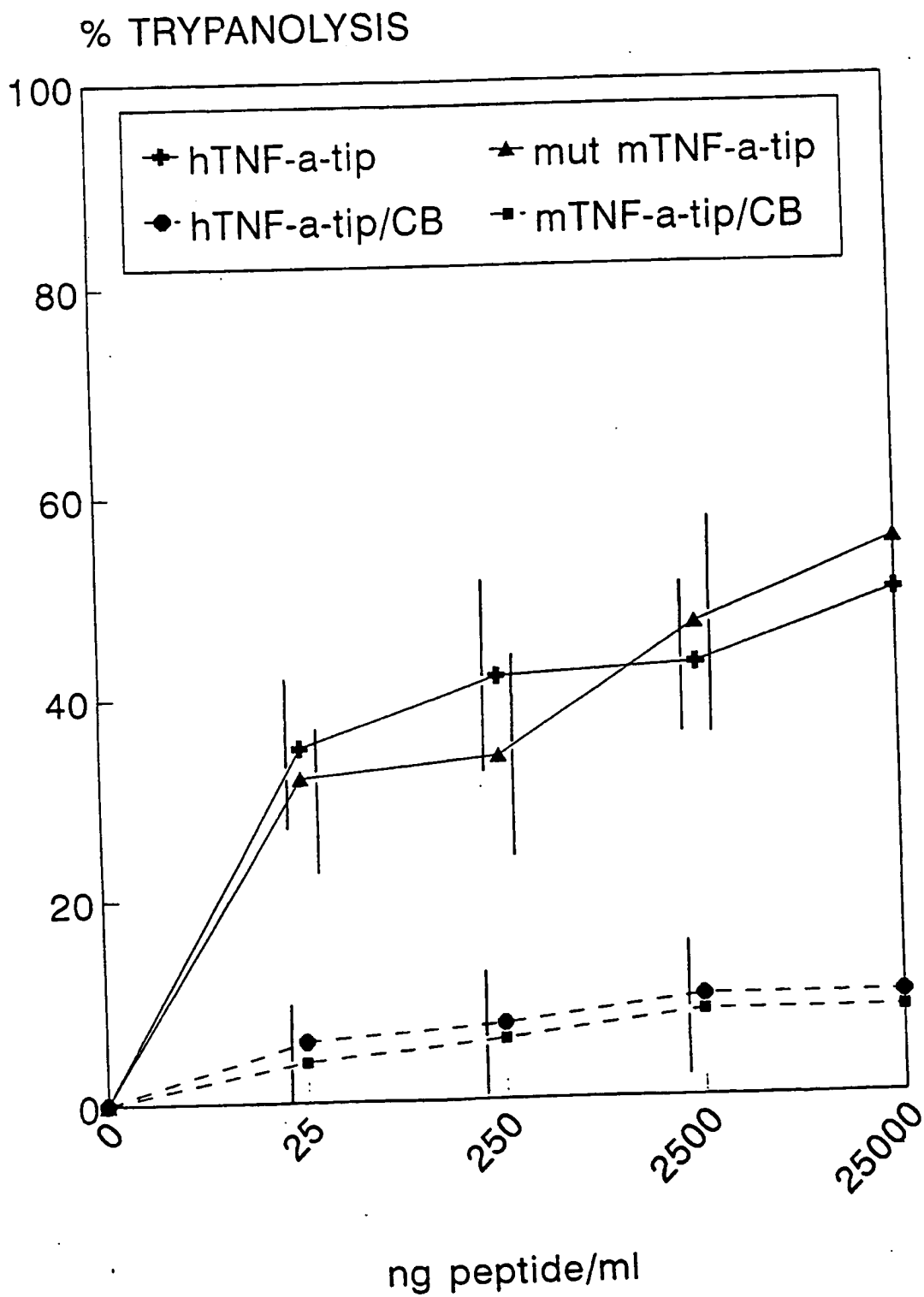


Figure 7

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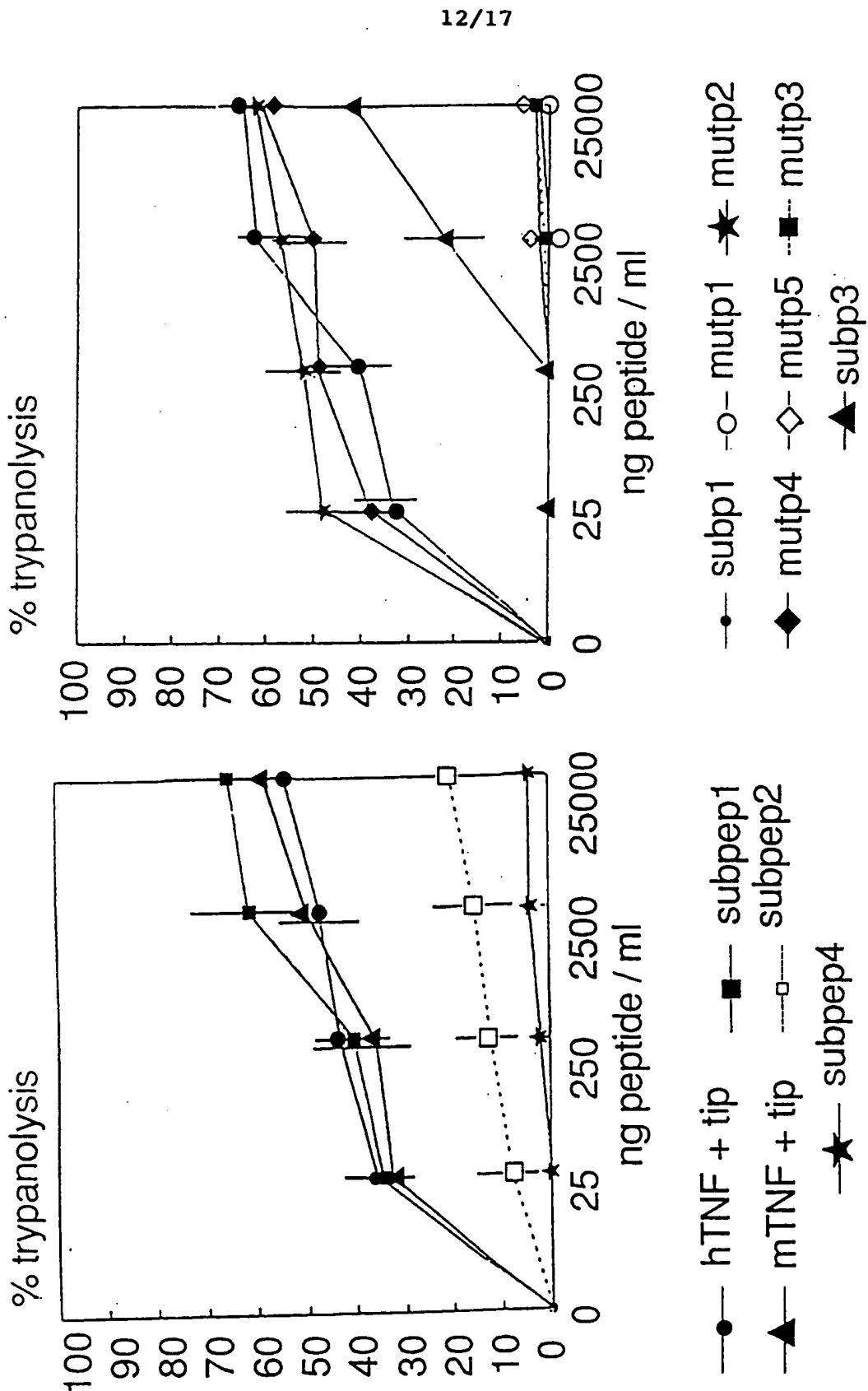


Figure 8

Figure 9

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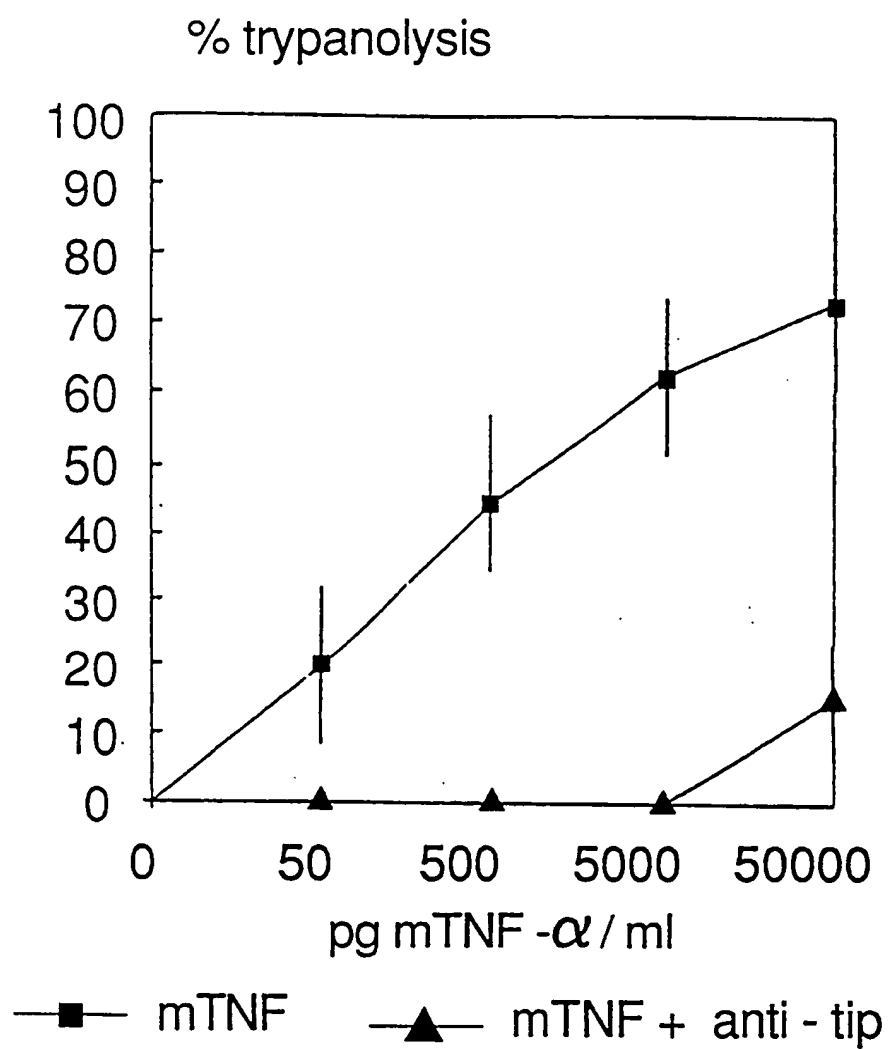


Figure 10

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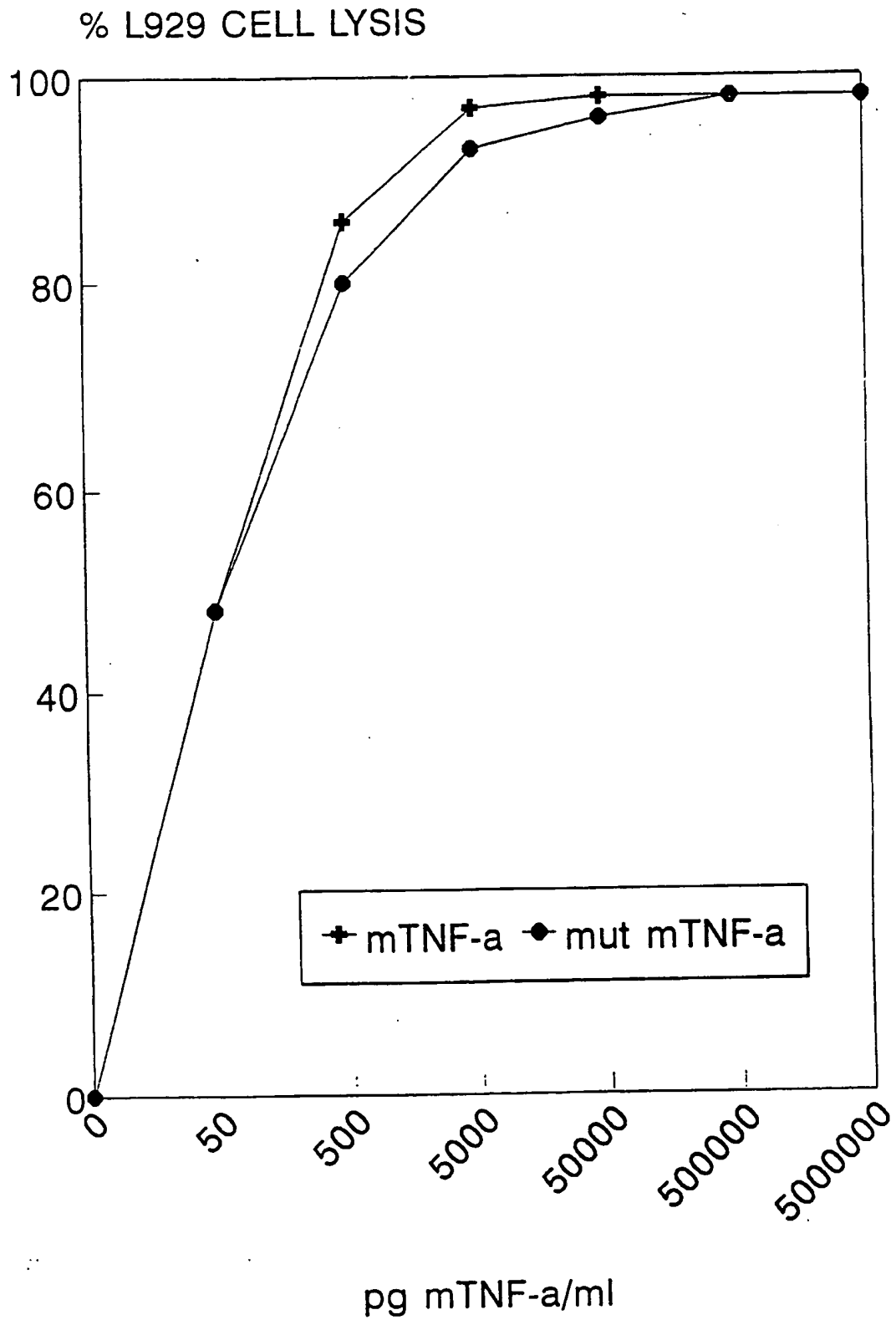
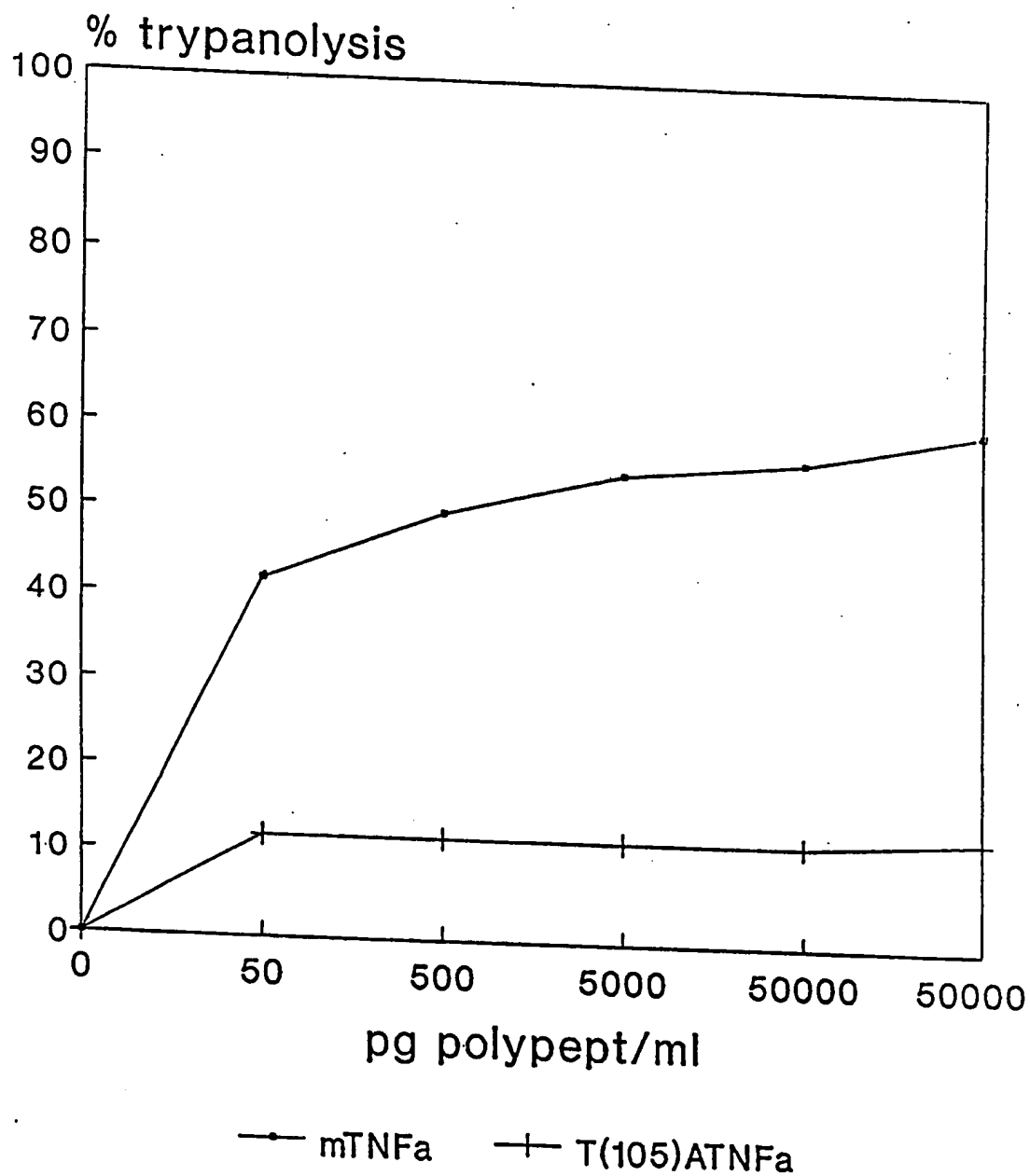


Figure 11

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trypanolytic activity of mTNFa and
T(105)A mutant after 5h



medium : PSG + 1% NMS

Figure 12
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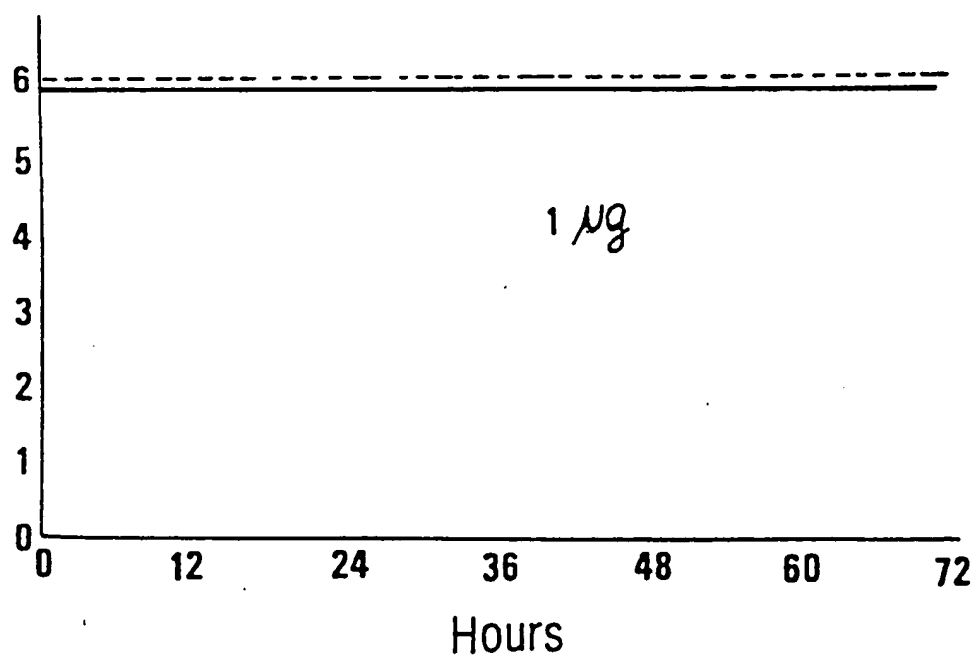
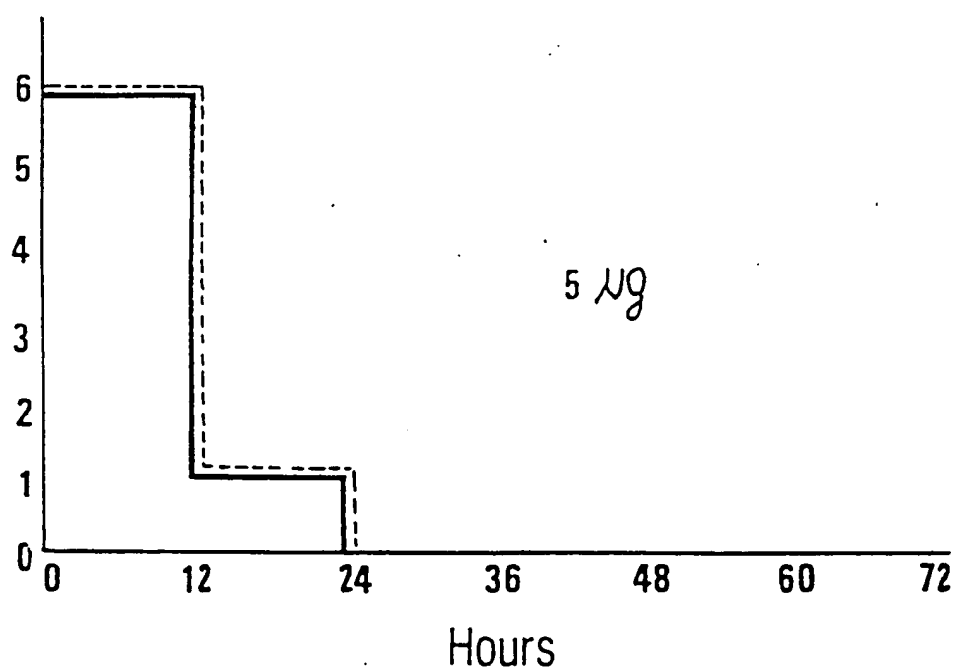


Figure 13

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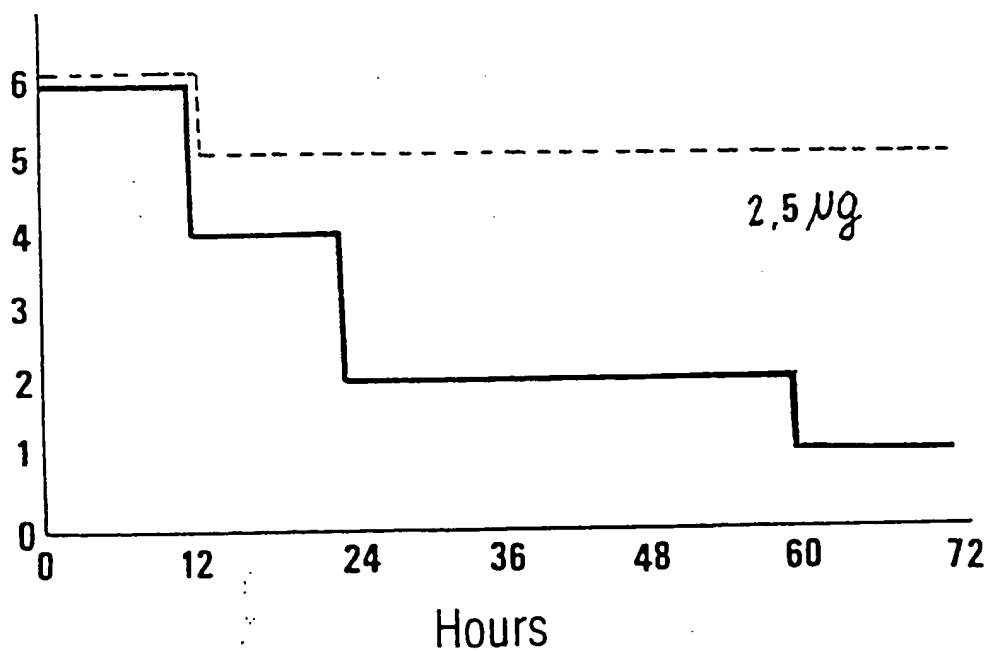
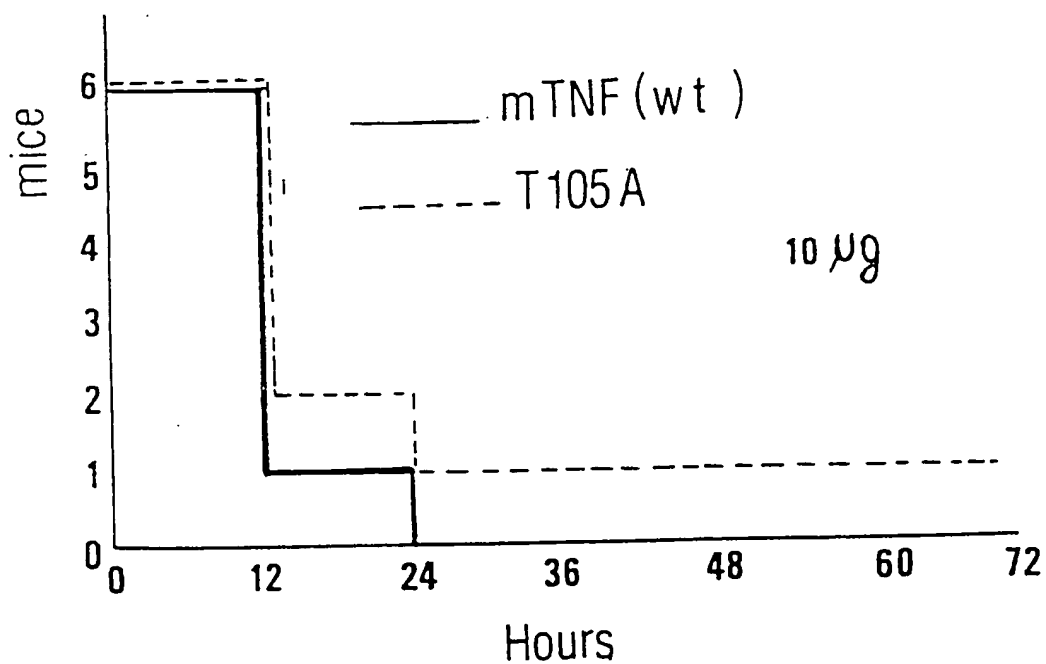


Figure 13 - continuation

INTERNATIONAL SEARCH REPORT

International Application No

PCT/EP 94/00286

A. CLASSIFICATION OF SUBJECT MATTER

IPC 5 C12N15/28 C07K15/00 A61K37/02 A61K39/395 C12N5/10
C12P21/02

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 5 C07K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	PROTEIN ENGINEERING vol. 3, 1990 pages 713 - 719 J. YAMAGISHI ET AL.; 'Mutational analysis of structure-activity relationships in human tumor necrosis factor-alpha' *abstract; Table II(a) and (b); discussion*	1, 18-24, 38-40
X	PROTEIN ENGINEERING vol. 3, 1990 pages 721 - 724 T. ARAKAWA ET AL.; 'Alteration in folding efficiency and conformation of recombinant human tumor necrosis factor-alpha by replacing cysteines 69 and 101 with aspartic acid 69 and arginine 101' *abstract*	1, 18-24, 38-40



Further documents are listed in the continuation of box C.



Patent family members are listed in annex.

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- "O" document referring to an oral disclosure, use, exhibition or other means
- "P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

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Date of the actual completion of the international search

13 June 1994

Date of mailing of the international search report

01. 07. 94

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Authorized officer

Yeats, S

INTERNATIONAL SEARCH REPORT

International Application No

PCT/EP 94/00286

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	EP,A,0 168 214 (GENENTECH, INC.) 15 January 1986 *page 15, line 32 - page 18, line 27; pages 63-66; claims* ---	1,18-24, 38-40
X	EP,A,0 479 071 (ISHIHARA SANGYO KAISHA, LTD.) 8 April 1992 *claims 1-19* ---	1,18-24, 38-40
A	EP,A,0 414 607 (ROUSSEL-UCLAF) 27 February 1991 *claims* ---	25
T	SCIENCE vol. 263 , 11 February 1994 pages 814 - 817 R. LUCAS ET AL.; 'Mapping the lectin-like activity of tumor necrosis factor' *whole document* -----	1-40

INTERNATIONAL SEARCH REPORT

International Application No
PCT/EP 94/00286

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
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		AU-B- 599571	26-07-90
		AU-A- 4465285	09-01-86
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		US-A- 5247070	21-09-93

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		AU-B- 642423	21-10-93
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		CA-A- 2023899	24-02-91
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		JP-A- 3091485	17-04-91
